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Conferences and Meetings

1978, Sept 21-24 First International Course on Multichannel Cochlear Implant to be held in Paris. Addr. Pr. C.-H. Chouard, Hôpital Saint Antoine 184, rue de Faubourg Saint Antoine, 75012 Paris

1978, Sept 25-30 The IVth Congress of the European Association for Maxillo-Facial Surgery (E.A.M.F.S.) will be held in Vicenza, Italy. Addr. Pavillon de Chirurgie Maxillo-Faciale et de Odontostomatologie Hôpital Régional "S. Bortolo", 36100 Vicenza, Italy

1978, Sept 25-29 The Third International Congress on Noise as a Public Health Problem: Biological and Behavioral Effects, is scheduled to be held in Freiburg West Germany. Addr. Int. Commission on Biological Effects on Noise, Universitätsklinikum, Johannes Gutenberg Universität, Obere Zahlbacher Strasse 67, D-6500 Mainz, BRD

1978, Oct 2-7 Cours Pratique de Septo-Rhinoplastie Fonctionnelle et de Rhinosinusoscopie donné à l'Université Libre de Bruxelles. Addr. Docteur Jacques Willemot, Lieutenant Willemotlaan 90, B-9910 Mariakerke Gent, Belgique.

1978, Oct 5-7 1st European Course in Pediatric Otolaryngology will be held in Trieste, Italy. Addr. Prof. R. Fiori, Istituto per l'Infanzia, Via dell'Istria 65, Trieste, Italy

1978, Oct 16-19 Cours Vestibulométrie Clinique donné en Strasbourg, France. Addr. Clinique O.R.L., Hospices Civils de Strasbourg, 1 Place de l'Hôpital, 67005 Strasbourg-Cedex

1978, Oct 30-Nov 1 Society for Neuroscience Eighth Annual Meeting will be held in Pittsburgh, PA. Addr. Dr. T. Gualtierotti, University of Pittsburgh, Vestibular Research Laboratory, 207 Hill Building, 3434 Fifth Avenue, Pittsburgh, PA 15260

1978, Oct 29 - Nov 3 The Vth International Conference on Pneumoconioses will be held in Caracas, Venezuela. Addr. International Labour Office, Occupational Safety and Health Branch, CH 1211 Geneva 22, Switzerland

1978, Oct 29-31 Semi-Annual VII Nerve Surgical Dissection Course of the New York Medical College will be held at Westchester County Medical Center. Addr. Mrs. P. Tamkin, c/o Dr. P. Guibor, 630 Park Avenue, New York, New York 10021

1978, Oct 30-Nov 10 A Two-Week Temporal Bone Surgical Dissection Course will be held at the Ear Research Institute in Los Angeles. Addr. Antonio de la Cruz, 256 South Lake Street, Los Angeles, CA 90057

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CONTENTS

<i>teller, A</i> (Stockholm, Sweden) Responses of Auditory Nerve Fibres to Noise Stimuli Show Cochlear Nonlinearities	
<i>oyoda, Y</i> and <i>Tachibana, M.</i> (Kyoto, Japan) Tissue Levels of Kanamycin in Correlation with Oto- and Nephrotoxicity	1
<i>achibana, M., Saito, H., Yamamichi, I</i> and <i>Morioka, H</i> (Kyoto, Japan) A Possible Involvement of Acidic Glycoaminoglycans in Kanamycin Oto-toxicity	9
<i>pusli, T., Hamrick, P. R</i> and <i>Walsh, P. J</i> (Research Triangle Park, USA) Ion Transport in Guinea Pig Cochlea	15
<i>gger Sjoberg, D</i> and <i>Wersall, J</i> (Stockholm, Sweden) Gentamicin induced Mitochondrial Damage in Inner Ear Sensory Cells of the Lizard <i>Calotes Versicolor</i>	22
<i>uko, M., Van de Water, T. R</i> and <i>Nordemar, H</i> (Stockholm, Sweden) Organ Culture of the 16th Gestation Day Mouse Labyrinth	35
<i>antos Saeghi, J</i> (New York, USA) Differential Effects of Primary Fixation with Glutataldehyde and Osmium upon the Membranous Systems of the Strial and External Sulcus Cells	52
<i>bpaka, G. R</i> and <i>Dubno, J. R</i> (Los Angeles, USA) Comments on the Acoustic reflex Response to Bone-conducted Signals	56
<i>Jackson, D. G</i> and <i>Lim, D. L.</i> (Columbus, USA) Fine Morphology of the Advancing Front of Cholesteatoma—Experimental and Human	64
<i>Fritze, W</i> (Vienna, Austria) A Computer-controlled Binaural Balance Test	71
<i>Virtanen, H</i> (Helsinki, Finland) Sonotubometry	89
<i>Remwall, U., Liden, G., Jungert, S</i> and <i>Nilsson, E.</i> (Goteborg, Sweden) Long term Observation of Ears with Reduced Middle Ear Pressure	93
<i>Karna, P., Jokipii, L., Ojala, K</i> and <i>Jokipii, A. M</i> (Helsinki, Finland) Bacteriology of the Chronically Discharging Middle Ear	104
<i>Brandt, Th., Allum, J. H. J</i> and <i>Dichgans, J</i> (Essen, BRD) Computer Analysis of Optokinetic Nystagmus of Peripheral Vestibular Origin	110
<i>Af elius, L. E., Henriksson, N. G</i> and <i>Wahlgren, L.</i> (Lund, Sweden) Vertigo as Reflected by the Nystagmogram	115
<i>Vinther, B</i> and <i>Elbrond, O</i> (Aarhus, Denmark) Nasal Mucociliary Function during Penicillin Treatment	123
<i>Illum, P</i> and <i>Balle, V</i> (Ålborg, Denmark) Immunoglobulins in Nasal Secretions and Nasal Mucosa in Perennial Rhinitis	132
<i>Schultz Coulon, H. J</i> (Hannover, BRD) The Neuromuscular Phonatory Control System and Vocal Function	135
	142

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CONTENTS

rg, E. (Stockholm, Sweden). Peripheral Vasoconstriction in the Rat in Response to Sound . . .	155
bezudo, Lucia M. (Boston, MA, USA) The Ultrastructure of the Basilar Membrane in the Cat . .	160
nishi, T. and Hamrick, P. E. (Research Triangle Park, NC, USA) Ion Transport in the Cochlea of Guinea Pig II. Chloride Transport.	176
right, C. G. and Hubbard, D. G. (Dallas, TX, USA) Observations of Otoconial Membranes from Human Infants	185
adson, H. C., Barnister, L. H. and Douek, E. E. (London, UK) Further Studies of the Effects of Continuous White Noise of Moderate Intensity (70-80 dB SPL) on the Cochlea in Young Guinea Pigs	195
uko, M. and Møller, A. R. (Stockholm, Sweden) A Physiological and Morphological Study of the Cochlea of the Rat Following Treatment with Minoxidil and Neomycin	201
inkel, O., Mørk Hansen, M., Kaaber, K. and Rozarth, K. (Hellerup, Denmark) A Prospective Study of Gentamicin Ototoxicity.	212
rus, K. C., Guth, P. S., Gwenz, G. D. and Burton, McKay C. (New Orleans, LA, USA) Fluorescent Staining of Hair Cells with Ethidium Bromide . .	217
ndersen, T., Skarstein, O. and Sikkeland, T. (Trondheim, Norway) A Study of the Vibration of the Basilar Membrane in Human Temporal Bone Preparations by the Use of the Mössbauer Effect	225
lva, T., Jauhainen, T., Sjöblom, C.-J. and Ylikoski, J. (Helsinki, Finland) Diagnosis and Surgery of Acoustic Tumours	233
dréansson, L., Harris, S. and Ivarsson, A. (Malmö, Sweden) Pulse Volume Recordings in outer Ear Canal in Pulse Synchronous Tinnitus	241
Meistrup-Larsen, K.-I., Mygind, N., Thomsen, J., Sørensen, H. and Vesterhauge, S. (Copenhagen, Denmark) Oral Norepinephrine in the Treatment of Acute Otus Media	248
Rubin, A. M., Lledgren, S. R. C., Ödqvist, L. M., Milne, A. C. and Fredrickson, J. M. (Linköping, Sweden) Labyrinthine and Somatosensory Convergence upon Vestibulospinal Neurons.	251
Schwarz, D. W. F., Tomlinson, R. D. and Milne, A. C. (Toronto, Canada) Directional Specificity for Head Rotation in the Pigeons Posterior Cerebellum	260
Palva, T., Horiling, L., Ylikoski, J. and Collan, Y. (Helsinki, Finland) Viral Culture and Electron Microscopy of Ganglion cells in Meniere's Disease and Bell's Palsy	269
Benedek-Spät, Erika (Budapest, Hungary) Sialochemical Examinations in Non-Tumorous Parotid Enlargements	276
Reimer, A. and Toremalm, N. G. (Malmö, Sweden) The Mucociliary Activity of the Upper Respiratory Tract.	283
Mogensen, Chr. and Tos, M. (Copenhagen, Denmark) Experimental Surgery on the Nose. . . .	289
Carenfelt, C., Lundberg, C., Nord, C.-E. and Wretling, B. (Stockholm, Sweden) Bacteriology of Maxillary Sinusitis in Relation to Quality of the Retained Secretion.	298
Axelsson, H., Carlöö, B., Welbring, J. and Winblad, B. (Umeå, Sweden) Aspergillosis of the Maxillary Sinus.	303

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CONTENTS

<i>bas W</i> (Vienna Austria) The Efferent Innervation in the Region of Inner Hair Cells in the Organ of Corti	309	<i>Mansson B</i> (Linköping Sweden) Tubal Closing Failure in Retraction Type Cholesteatoma and Adhesive Middle Ear Lesions	408
<i>iss M</i> (Ann Arbor USA) Glycogen Accumulation in Reissner's Membrane Following Chemical Sympathectomy with 6-Hydroxydopamine	314	<i>Hotho T., Kato I., Kodama A and Suzuki H</i> (Hamamatsu, Japan) Sudden Deafness in Relapsing Polychondritis A Scanning Electron Microscopy Study	418
<i>oldřich L.</i> (Prague Czechoslovakia) Mechanical Properties of Basilar Membrane	331	<i>Margolis R. H., Osguthorpe J. D. and Popelka R.</i> (Los Angeles, USA) The Effects of Experimentally Produced Middle Ear Lesions on Tympanometry in Cats	42
<i>usaka J., Kambayashi J., Ise I. and Kawamoto K</i> (Sendai Japan) Reduction of the Endocochlear Potential by the new "Loop" Diuretic, Bumetanide	336	<i>Engell U., Englund A. and Westerholm P</i> (Stockholm, Sweden) Nasal Cancer Associated with Occupational Exposure to Organic Dust	437
<i>John G. M. and Fermin C. D.</i> The Development of Hair Cells in the Embryonic Chick's Basilar Papilla	342	<i>Uddman R., Almqvist J., Densert O., Halanson, R. and Sundler F</i> (Malmö Sweden) Occurrence and Distribution of Visceral Nerves in the Nasal Mucosa and Tracheobronchial Wall	443
<i>Thomsen J., Bech P. and Saxtrup Nielsen, O</i> (Copenhagen, Denmark) CSF Total Protein Normal Values	359	<i>Torgjussen W., Haug F. M. S. and Andersen I.</i> (Oslo Norway) Concentration and Distribution of Heavy Metals in Nasal Mucosa of Nickel Exposed Workers and of Controls, Studied with Atomic Absorption Spectrophotometric Analysis and with Timm's Sulphide Silver Method	449
<i>Henry K. R. and Lepkowski C. M.</i> (Davis, USA) Evoked Potential Correlates of Genetic Progressive Hearing Loss	366	<i>Evans R.</i> (Cardiff Great Britain) The Central Rhythm of the Nasal Cycle	464
<i>Kaneko Y., Terayama Y., Kawamoto K., Kasayama K. and Ise I.</i> (Sapporo Japan) Single-Cell Layer Membrane Covering the Degenerated Cochlear Duct after Perilymphatic Perfusion of Streptomycin	375	<i>Sege G.</i> (Erfurt, GDR) Characterization of Clinical Tonsil Stages by their T-Cell Count Immunopathological Description of Tonsils after a Peritonsillar Abscess	465
<i>Humes L. E. and Bass F. H.</i> (Nashville USA) A Test Battery Approach to the Investigation of Susceptibility to Temporary Threshold Shift	385	<i>Grönroth O., Hellquist H., Olofsson J. and Nord</i>	47
<i>Brown, J. J., Brummett R. E., Meikle M. B. and Vernon, J.</i> (Portland, USA) Combined Effects of Noise and Neomycin Cochlear Changes in the Guinea Pig	394		
<i>Virtanen H.</i> (Helsinki, Finland) Patulous Eustachian Tube	401		

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RESPONSES OF AUDITORY NERVE FIBRES TO NOISE STIMULI SHOW COCHLEAR NONLINEARITIES

Aage R. Møller

*From the Division of Physiological Acoustics, Department of Environmental Medicine
Karolinska Institutet, Stockholm, Sweden*

(Received October 6 1977)

Abstract The frequency selectivity of the peripheral auditory frequency analyser was determined on the basis of recordings from single auditory nerve fibres in the rat using pseudorandom noise as stimulus. The impulse response function was arrived at by cross-correlating period histograms of the recorded activity with the noise. Frequency transfer functions were computed by Fourier transformation of the derived impulse response function. The impulse response function was observed to become shorter with increasing stimulus intensity. A concomitant widening of the frequency transfer function and a downward shift in the centre frequency were seen. Since the method used is insensitive to nonlinearities, the derived impulse and frequency response functions are assumed to be valid approximations of the basilar membrane properties. The results then support the hypothesis that the basilar membrane is a nonlinear frequency analyser whose selectivity decreases as sound intensity increases.

The model generally accepted for the peripheral auditory analyser is a band pass filter implemented by a rectifier, a lowpass filter and a triggering device. The band pass filter consists of the frequency selectivity of the basilar membrane, the rectifier is the unidirectionality of the sensory hair cells and the low pass filter is the smoothing action of the neural excitatory process that transduces the vibration of the hair cells into nerve impulses in the axons of the auditory nerve (Weiss 1966).

A fundamental question is whether the frequency selectivity of the auditory periphery is solely a result of mechanical tuning of the basilar membrane. The first quantitative data on basilar membrane tuning originate from v. Bekesy who measured the displacement of the basilar membrane of human cadaver ears in the low frequency region of the basilar mem-

brane. These results represented a great step forward in auditory physiology but at the same time they emphasized the need of an additional mechanism in order to explain the great frequency selectivity of the ear. When it became possible to measure basilar membrane vibration at lower sound intensities and in anesthetized animals, as has been done by Rhode (1971), Johnstone et al. (1970) and Wilson & Johnstone (1972), greater frequency selectivity values (narrower tuning curves) were obtained than those presented by v. Bekesy. The obtained mechanical tuning curves of the basilar membrane were, however, wider than frequency threshold curves of single auditory fibres. Such frequency tuning curves (FTC), show the threshold of single nerve fibres in response to pure tones as a function of frequency while the mechanical tuning curves show the vibration amplitude at or above 70 dB SPL (Rhode, 1971). A fundamental question is whether this discrepancy in frequency selectivity is due to the fact that the vibration of the basilar membrane is nonlinear in such a way that the selectivity increases as sound intensity is decreased.

The observation by Rhode (1971, 1973) shows that the frequency selectivity of the basilar membrane in the squirrel monkey decreases with sound intensity between 70 and 90 dB SPL. That could account for the dis-

Present address: Eye & Ear Hospital, University of Pittsburgh School of Medicine, Pittsburgh, Pa. 15213, USA.

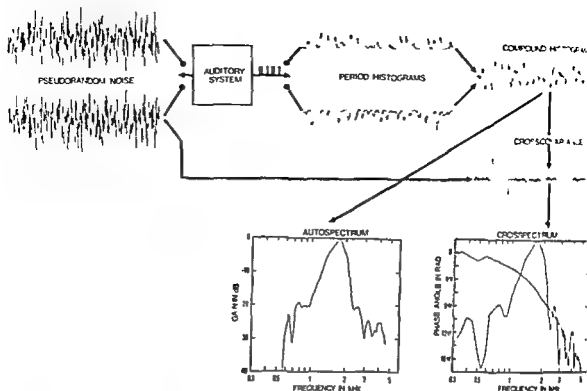


Fig. 1. Schematic illustration of the data processing.

crepancy between neural and mechanical tuning. However, other studies using similar or different methods (Johnstone et al., 1970; Willott & Johnstone, 1972) for measuring basilar membrane vibration in the guinea pig have failed to show such nonlinear motion.

Various types of mechanical transformations of the motion of the basilar membrane are therefore still further explanations which might be proposed for the discrepancy in the sharpness of the frequency tuning observed between the neural tuning curves and the motion of the basilar membrane. The basilar membrane motion has always been measured in terms of up and down movement. It is not certain that the up and down movement of the basilar membrane is the most efficient stimulus for the hair cells.

Evans (1975) assumes another explanation. He developed the hypothesis that there is a sharply tuned (presumably neural) band pass filter succeeding the relatively broadly tuned cochlear (mechanical) filter. He found that the existence of such a "second filter" was sup-

ported by the results of experiments on animals with normal cochleas (Kiang et al., 1965; Evans, 1974a, b). Evans (1975) thereby concluded that the neural threshold tuning curves were sharper and narrower than the mechanical tuning curves of the basilar membrane, owing to this "second filter".

The technical difficulties inherent in measuring the basilar membrane motion directly at physiological sound levels make it appealing to use neurophysiological methods to determine basilar membrane displacement, whereby hair cells serve as "sensors" of the basilar membrane vibration.

Kiang et al. (1965) showed that peristimulus time histograms of the response of peripheral auditory nerve fibres with a low characteristic frequency had a periodic pattern, assumed to reflect damped oscillation of the basilar membrane. Unfortunately, such results do not lend themselves to a quantitative analysis, mainly due to the influence on the responses of the nerve

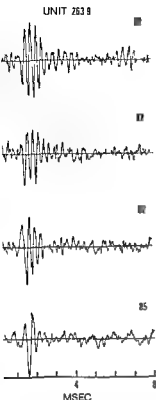


Fig. 2. Typical cross-correlograms representing the impulse response function of a typical nerve fibre. The stimulus intensities (dB SPL, measured in 1/3 octave steps) are indicated by caption numbers. The threshold of the unit was about 15 dB SPL. The amplitude of the correlograms is normalized to the same maximal value.

properties of the neurone involved. The nonlinearity resulting from the neural transduction in the hair cells also influences the results.

Libick & Pfeiffer (1969) further developed the above mentioned method and introduced a correlation procedure to disclose nonlinearities in the inner ear. They combined the histograms of the responses to clicks of one polarity with those of the responses to the clicks of the reverse polarity, which yielded a combined histogram assumed to be an approximation of the basilar membrane in both directions. Although this method provided important information about the nonlinearities in the cochlea, it did not enable the determination of the impulse response function of the basilar membrane. Other studies (Møller, 1970) have

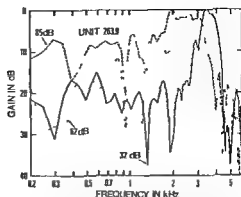


Fig. 3. Cross spectra of the responses of the unit depicted in Fig. 2 representing the frequency domain transfer functions. The cross spectra are normalized to the same maximal value.

shown that the frequency selectivity represented by neural tuning curves is available immediately. A neural sharpening involving conventional chemical synaptic transductions can therefore be excluded since that would result in delays on the order of at least 0.7 msec.

Quantitative information about the spectral acuity of the auditory frequency analyser at different stimulus intensities was hence lacking.

The discharge pattern of single auditory fibres in response to noise stimuli can provide quantitative information about the spectral selectivity of the auditory periphery. De Boer (1969, 1970), using a reverse correlation technique, found that the frequency selectivity of single primary nerve fibres of the cat in response to band-pass filtered noise was similar to the frequency threshold curves (FTC) obtained in response to pure tones.

The reverse correlation technique is similar to cross correlating the output of a system with the input. When the input is a random noise with a uniform spectrum in the frequency range where the system under test has significant transmission, the cross correlation function is a valid approximation of the system's impulse response. The results are essentially independent of nonlinearities (memoryless) that follow the frequency selective filter of the system. When this method is

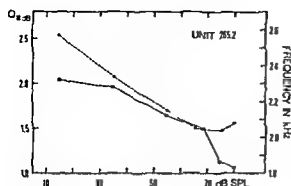


Fig. 4. Relative width (Q_{10dB} ●---●) and center frequency (■—■) of the transfer function plotted as a function of the sound level (in dB SPL, measured in 1/3 octave bands).

applied to a system in which the input first enters a linear band pass filter and then a non-linear device the results largely reflect the linear properties of the system. If it can be assumed that the discharge rate of single auditory nerve fibres is a nonlinear transformation of the vibration of the basilar membrane this method would thus yield primarily the linear properties of the membrane including the operation of Corti. It is also necessary that the non-linearity of the neural transduction can be regarded to be memoryless. Using continuous noise also renders the obtained latency values independent of the stimulus intensity (Møller, 1975) compared with using click stimulation where the latency is a function of the sound level.

In the present study the impulse response function of the peripheral auditory analyser was determined on the basis of the responses of single auditory nerve fibres in the anesthetized rat.

METHODS

White rats weighing 200–300 g anesthetized with Urethan (1.5 g/kg bw) were used. The occipital part of the skull was opened and parts of the cerebellum were removed by suction to render the cochlear nucleus visible. The pinnae were deflected and the ear canal cut after which the head was fixed in the headholder with hollow earbars. For further details on

this procedure were given in previous publications (Møller, 1969). Recordings were from single auditory nerve fibres using fine glass pipettes filled with 2 M sodium acetate and with a resistance of $M\Omega$. The electrodes were placed in contact with the surface of the dorsal cochlear nucleus aiming at the auditory nerve's entrance skull cavity (Møller, 1976). Pseudorandom noise was used as stimulus. It was generated by a Hewlett Packard noise generator 3722 A and applied to a condenser microphone (Bruel & Kjaer type 4131) via electronic amplifiers and attenuators. The condenser microphone was connected to the low earbars of the headholder (see Møller, 1969). The noise generator was set to a period of 3.33 μ s and a sequence length of 4095 points, i.e. each noise period had a duration of 13.64 msec. The noise was low-pass filtered with an upper cut off frequency of 10 kHz (see Møller, 1974, 1976, 1977).

Period histograms were produced by an Inter technique DIDAC 800 synchronized with the periodicity of the noise with a width of 20 μ s. The polarity of the noise was reversed during the recording every 100 points and the summation of the discharge rate was changed to subtraction to produce com-

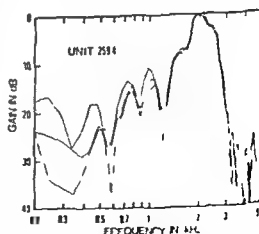


Fig. 5. Cross spectra of the responses of the auditory nerve immediately after the unit was encountered and 70 minutes later. The sound intensity was 70 dB SPL and each curve is based on 2 minutes of data.

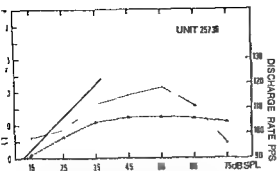


Fig. 6A Average discharge rate (○ ○) and relative peak value of the computed cross spectra (▲—▲) as a function of stimulus sound level (in dB SPL measured 1/3 octave bands)

ograms (see Fig. 1). The histograms were then processed off-line to produce auto-covariance

produce auto, cross and coherence spectra (see Fig. 1 and Møller, 1974, 1976)

RESULTS

The results of the present study are based on recordings from 52 fibres. Fig. 2 shows typical cross correlation functions estimated from discharges in response to noise with intensities ranging from just above threshold (32 dB SPL) to 85 dB SPL (measured in a 1/3 octave band). The cross correlation functions were normalized to the same peak value.

The duration of these correlation functions is seen to decrease as the sound intensity increases, indicating that the bandwidth of the auditory analyser is narrower for low than for high intensity sounds. The computed frequency transfer functions (cross spectra) in Fig. 3 show even more clearly that the tuning curve widens at high stimulus intensity than at low. In addition to the widening of the tuning curve, the centre frequency is shifted downwards as stimulus intensity is increased.

The quantitative relationship between the relative width of the peak of the frequency transfer function and the sound intensity is

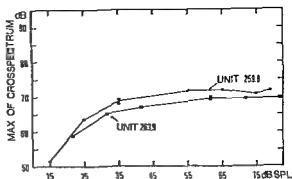


Fig. 6B Relative peak value of the cross spectra of two other units

seen in Fig. 4, where the centre frequency is also plotted. The relative width is expressed as Q_{10dB} , i.e., the ratio between the centre frequency and the bandwidth measured at 10 dB below the peak.

The Q -values are observed to decrease monotonically throughout the entire range of intensities studied, i.e., from about 10 dB above threshold to more than 80 dB above threshold. The centre frequency is also seen to decrease monotonically within about the same range of intensities.

The cross spectra (transfer functions) seen in Fig. 3 are normalized with regard to the peak value amplitudes. Fig. 5 shows the peak value of the transfer function as a function of the stimulus intensity. The peak value of the transfer function thus determined is a measure of the average degree of phase locking of the discharges to the sound stimulus filtered by the basilar membrane. The peak value of the transfer function is noted to reach a plateau at only about 30 dB above the threshold after which it remains virtually constant throughout the intensity range studied. Fig. 5 also depicts the average discharge rate as a function of the stimulus intensity. This curve follows a course that is somewhat different from that of the peak value of the transfer function.

These results, typical for units with a centre frequency between 1 and 4 kHz, show that the peripheral auditory analyser functions as a nonlinear spectrum analyser in response

to noise stimuli. Since the cross-correlation method used is assumed to be uninfluenced by nonlinearities occurring after the frequency selective elements, the nonlinearity is not likely to originate from the neural transduction process. Its source is therefore assumed to be in some activity which would take place prior to the mechanical excitation of the hair cells. The nonlinearity found in the present study closely resembles those shown by Rhode in the vibration of the basilar membrane (Rhode, 1971, 1973) in the intensity range from 70 to 90 dB SPL. It may therefore be inferred that the nonlinearities in the present study also originate in the motion of the basilar membrane. Moreover, the present results indicate that the nonlinearity extends to lower intensities, thereby bringing about a narrowing of tuning towards lower sound levels. Near threshold the frequency transfer functions obtained using noise stimuli are nearly identical to frequency threshold tuning curves obtained using pure tones as stimuli. It seems as though the selectivity in single auditory nerve fibres, at least in a first approximation, may be explained on the basis of the vibration of the basilar membrane. Results of earlier studies, showing that the full frequency selectivity of auditory neurons is available with no measurable delay (Møller, 1970), are thus supported. The sharp tuning seen at low intensities is likely to have little to do with a neural sharpening of a broad mechanical tuning of the basilar membrane.

Finally, it may be inferred from the present results that the waveform of the filtered version of a broadband stimulus is reproduced in the discharge pattern of single auditory nerve fibres within a much larger intensity range (more than 60 dB) than that within which the average discharge rate is a function of the intensity of a continuous tone. The mean discharge rate usually reaches a saturation level at only about 20–30 dB above threshold. In other studies the discharge rate of single auditory nerve fibres has usually been found to saturate only 20–30 dB above threshold.

These findings, too, are consistent with the results of the present study.

The dynamic range of the method used for obtaining the cross-spectra is estimated slightly more than 20 dB. Thus spectral values below 20–25 dB are not really significant in the meaning of the finer details in the complex cross spectra (peaks and valleys) seen, particularly below the frequency of the maximum. This is not clear. However, the reproducibility of these cross-spectra is good, as illustrated in Fig. 5 where the cross-spectra obtained from the same fibre in three successive recordings are shown. Even the peaks below the frequency of the maximum value are reproduced in the spectra computed from all three recordings.

The results graphed in Fig. 6 indicate that the number of phase-locked discharges is remarkably constant in the larger part of the dynamic range of the auditory system. The phase-locked responses of auditory nerve fibres are thus to be regarded as an amplitude-compressed version, and heavily so, of the motion of the basilar membrane. However, there is little information as to the absolute values of the sound intensity in the discharge pattern of primary fibres. Nonetheless, such an amplitude-compressed version of the basilar membrane motion seems extremely well suited as a basis for temporal analysis. Basilar membrane filtering, in the frequency range where phase locking is prominent, may be assumed to mainly to separate spectral components whereupon masking is avoided as a prerequisite for a temporal analysis. According to this line of reasoning, it seems as though temporal analysis may be more important than spectral analysis as a basis for frequency discrimination in the peripheral auditory system. If such is the case, perhaps the cochlea does not take on its role as a spectral analyser for frequency discrimination until we are dealing with frequency ranges beyond that within which phase locking is prominent.

Decoding of spatial frequency information requires that the nerve fibres be identified

higher auditory centre with regard to average discharge rate while decoding of oral frequency information calls for the modulation, somewhere in the central nervous system, of the modulation of the intervals between individual discharges. We do not know how the periodicity information of a periodic sound is extracted, but neurophysiological evidence indicates that the differences are required for a sound to reach the two can be extracted. It is possible that a similar principle is followed in analysis of aural periodicities whereby the phase-locked responses in individual auditory nerve fibres would serve as a basis for this analysis. The result would then be a decoding into another form elsewhere in the auditory nervous system (see Møller, 1978).

The present results, obtained in rats as they were, can be regarded as representative of the human auditory system. It seems as though the basic principles for frequency analysis in the inner ear differ depending upon whether the frequency is above or below 4-5 kHz. In this connection, it is worth noting that the spectral components of human speech are the important factor for discrimination below 4 kHz. It is likely, then, that both formant frequencies and the fundamental frequencies of vowels and other voiced sounds are determined on the basis of the temporal analysis in the inner ear.

In patients with hearing loss due to cochlear damage, Pickett & Danaher (1973) have shown that the ability to discriminate small changes in the frequency of the second formant (F_2) of a two-formant synthetic vowel was impaired while the ability to discriminate frequency changes in a single formant with the same centre frequency as F_2 was nearly normal. They suggested that this decrease in frequency discrimination may be due to an increased degree of masking from the first formant resulting from the cochlear damage. On the basis of the results of the present study, it may then be inferred that a broadening in the basilar membrane tuning increases the

masking of information, when we are dealing with low frequencies, whereby temporal analysis is impaired.

Discrimination of sound intensity cannot be explained on the basis of the above described model. Since the dynamic range of single nerve fibres with regard to mean discharge rate as well as number of phase locked discharges is small, only about 20 dB, information on sound intensity throughout the entire auditory range cannot be communicated by the discharge rate of individual fibres alone. Intensity information needs only a few nerve elements and may be taken care of by a few nerve cells receiving input from a large number of auditory nerve fibres and located in higher brain nuclei, such as the cochlear nucleus. Through integration of the output of many primary fibres, information about the absolute sound intensity may be obtained.

ACKNOWLEDGEMENT

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ZUSAMMENFASSUNG

Die Frequenzselektivität des peripheren Frequenzanalysators des inneren Ohres wurde an Hand von Mikroelektrodenmessungen von einzelnen Fasern des Nucleus der Ratte bestimmt. Als Reiz diente pseudo-stochastisches Rauschen. Die Impulsantwort wurde durch Kreuzkorrelation der Periodenhistogramme der bezeichneten Aktivität mit dem Rauschen erhalten. Mit Hilfe von Fourier Transformation der Impulsantwort wurden dann die Frequenzübertragungsfunktionen berechnet.



Es stützen damit die Hypothese, daß die Basilarmembran einen nichtlinearen Frequenzanalysator darstellt, dessen Selektivität mit wachsender Tonintensität abnimmt.

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TISSUE LEVELS OF KANAMYCIN IN CORRELATION WITH OTO AND NEPHROTOXICITY

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Abstract The time courses of kanamycin (KM) levels in perilymph, kidney, liver, brain and blood were observed by a bioassay method in normal guinea pigs and in mice pretreated with KM for 10 successive days. In the kidney and perilymph of the normal guinea pigs, the injected KM became highly concentrated and was eliminated only after a protracted delay. This tendency was much more intensified in kidney and perilymph of the pretreated animals. By contrast, the KM in blood and brain is eliminated rapidly both in the normal and in the pretreated animals. It is concluded that the high accumulation and slow elimination of KM by the inner ear and brain, and moreover the enhancement of these phenomena by successive pretreatment are one of the important factors in the origin of oto- and nephrotoxicity.

The streptomycetes antibiotics, such as streptomycin and kanamycin (KM), are indispensable even today for treatment of tuberculosis or pseudomonas infections despite their oto- and nephrotoxicities. To avoid these toxicities, it is essential to understand their action mechanisms. The studies of Vrabec et al (1965), Oldrich (1965) and Stupp et al (1967) revealed that the toxic streptomycetes antibiotics became highly concentrated in the cochlea and are eliminated from there only after a considerable period of time. The genesis of the ototoxicity has been explained on the basis of these points.

However, the following questions still remain to be answered in order to explain the selective mechanism of their toxicities to the inner ear and the kidney.

Are the accumulative propensity and delay elimination of the drugs specific to the inner ear as opposed to other organs?

How does the repeated administration affect

the processes of accumulation and elimination of these drugs in the various organs?

We investigated the time course of the concentration of the streptomycetes antibiotics in the selected organs and blood before and after repeated administration. We selected the cochlea and kidney to serve as the model for the vulnerable organs and the brain and liver for the non vulnerable organs. We used kanamycin as test antibiotic, since no essential differences from other streptomycetes antibiotics have as yet been reported for tissue concentration.

METHOD

Sixty-six healthy albino guinea pigs weighing 300-400 g were divided into two equal groups at random. The first group was the untreated control group. The second group was pretreated with KM (400 mg/kg b.w.) for 10 successive days and sacrificed 48 hr after the last injection. The animals of both groups were given KM (400 mg/kg b.w.) intramuscularly and sacrificed 1, 3, 6, 12 and 24 hr subsequently. Several animals of both groups were sacrificed without KM injection, for control purposes.

The chests of the animals were opened under ether anesthesia and the blood was aspirated from the hearts. After decapitation, the temporal bone, brain, liver and kidney were removed. To collect perilymph from the opened bulla, a thin glass capillary was introduced through the oval and the round win-

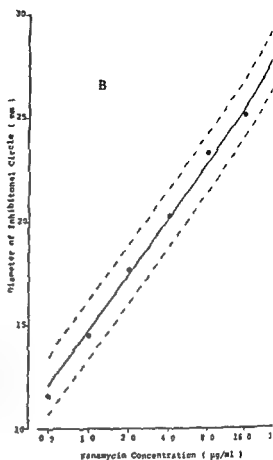
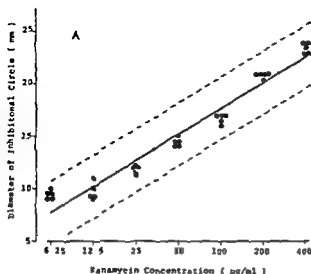


Fig 1 Standard curves of KM concentration in the function of the diameters of inhibitory circles of bacterial growth by known concentrations of standard KM solution in the cup method (A) and in the disc method (B). Each circle represents the mean values of 5 samples (A) or one value (B) — obtained by linear regression --- 95% confidence limits.

ows into the scala tympani and the scala vestibuli, respectively. The aspirated perilymph was examined under a microscope and any samples contaminated with blood were discarded.

The brain, liver and kidney were homogenized and the homogenate or the whole blood was diluted with 0.1 mole phosphate buffer (pH 7.0) to the required antibiotic concentration.

The agar diffusion method for the determination of KM was applied in the form of a cup test for blood and tissues and a disc test for perilymph. A filter leaf No. 50 (Toyo Roshi) with a diameter of 5 mm was used. *Bacillus subtilis* ATCC 6633 served as test bacterium. The minimum detectable range of KM was 0.01 µg/ml in the cup test and 0.5 µg/ml in the disc test. The standard curves of the two methods are shown in Fig. 1.

RESULTS

Kanamycin levels in the normal animals

Fig. 2 shows the time courses of KM level in the various tissues. The minimum value before KM injection represents certain non-specific antibacterial activities, such as lysozyme activity. The maximum KM level in the tissues was highest in kidney (828 ± 48) followed by blood (361 ± 29), perilymph (41 ± 4), liver (36 ± 5) and brain (6 ± 1). Each value in parentheses represents the mean (microgram/ml or g w w ± S.E.). These maximum values were reached at 1 hr after injection—except that in the perilymph, which appeared at 6 hr. After 6 hr, the KM level in the blood, liver, and brain decreased to the low level of <10 µg/ml g w w. However, the level in kidney and perilymph remained high. The KM level in liver, blood, and brain showed a tendency toward

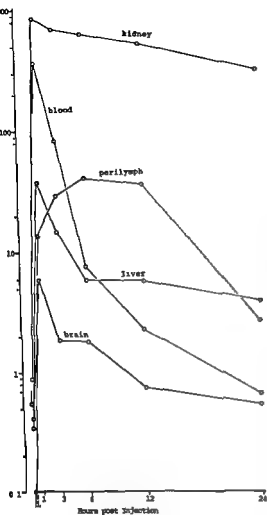


Fig. 2 The time courses of KM levels in the various tissues and fluids of the control group. Each circle represents the mean value obtained from varying numbers of samples as indicated in Figs. 3-8.

rapid elimination, while that in perilymph and kidney showed a tendency to prolonged existence.

Kanamycin levels in the pretreated animals

The KM levels in each tissue were compared between the control group and the KM pretreated group and evaluated statistically by the student's *t* test.

Figs. 3 and 4 show the time course of the KM level in blood and brain, for both groups.

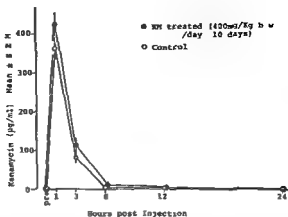


Fig. 3 In Figs. 3-8 the time courses of KM levels in the selected tissues and fluids are compared between the control group (○) and the pretreated group (●). These are the time courses of KM levels in the blood. Each value represents the mean and S.E. obtained from 6 samples. No significant difference in values was observed between the two groups at any time.

No significant change was observed at any time in blood and brain. The ratios of maximum KM levels in blood and brain of the pretreated group vis-à-vis those of the control group were 0.86 and 0.62, respectively. And the ratios of KM levels in blood and brain at 24 hr in pretreated group vis-à-vis those in the control group were 0.41 and 0.62, respectively.

In contrast, as shown in Figs. 5, 7 and 8, the KM levels in perilymph of the scala vestibuli and the scala tympani in the kidney, and in the liver of those in the pretreated group were significantly higher than those of control group at the indicated times. The ratios of maximum KM levels in the pretreated group vis-à-vis those in the control group were about 1.07, 1.64, 1.83 and 2.43, respectively. The ratios of KM levels at 24 hr in the pretreated group vis-à-vis those in the control group were about 5.71, 12.21, 4.34 and 8.67 in each tissue, respectively. These ratios mean that KM accumulated more intensively in and was eliminated more slowly by those tissues of the pretreated group than by those of the control group. Furthermore, the peak KM level in the perilymph, especially in that of the scala vesti-

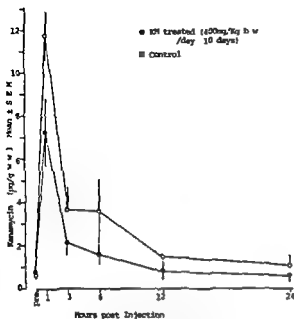


Fig 4 The time courses of KM levels in the brain. Each value represents the mean and S.E. obtained from 6 samples. No significant difference of the values was observed between the two groups at any time.

built, of the pretreated group occurred earlier than that of the control group.

DISCUSSION

The results show that the behavior of KM in the inner ear and the kidney is unique to the other organs. The high KM concentrations in these tissues were accompanied by an increase in blood level but remained at a high level even after KM in blood had been almost eliminated. Furthermore this tendency in the inner ear and the kidney, i.e. "high accumulation" and "slow elimination", were enhanced by repeated treatment of the same antibiotics, in contrast to the findings of Stupp (1970). Since these phenomena were not observed in blood and brain, they should correlate to the origin of oto- and nephrotoxicity. However the toxic mechanism of KM cannot be entirely explained by these phenomena alone, since the same kind of phenomenon even though not so remarkable, was also observed in the liver, which was believed not to be influenced by KM. Some other cause of specific organ

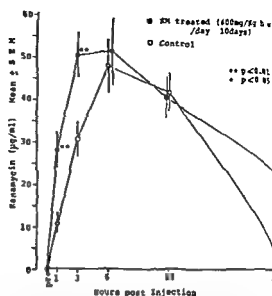


Fig 5 The time courses of KM levels in the scala tympani. Each value represents the mean and S.E. obtained from 6 to 12 samples. The values of the pretreated animals are significantly higher than those of the control animals at the times and *p* values indicated by asterisks in Figs 5-8.

toxicity needs to be considered in order to explain the specific vulnerability of the sensory hair cell in the cochlea and the urinary tubules to KM (Tachibana, 1976). The above mentioned specific behavior of KM in the

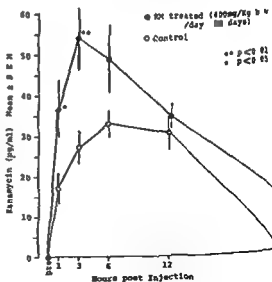
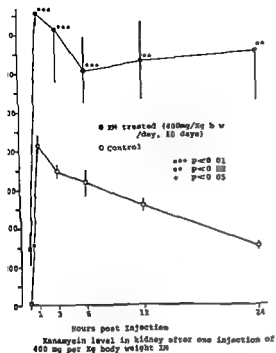


Fig 6 The time courses of KM levels in the scala vestibuli. Each value represents the mean and S.E. obtained from 6 to 12 samples.



7 The time courses of KM levels in the kidney; value represents the mean and S.E. obtained from 6 samples

chlea and kidney, however, is considered to be a factor of primary importance for the oto- and nephrotoxicities. To understand the mechanism in the cochlea we need to know the penetration and elimination routes of the antibiotics in this tissue. Though the complete answer to this question is not yet available, the tentative site of the penetration and elimination is, on morphological premises, considered to be the plexus cochlearis and the lateral wall of the cochlea. There is no evidence that the lateral wall has any mechanism for the active elimination of streptomycin antibiotics from the perilymph. However, Saito et al. (1976) demonstrated that the lateral wall contained a greater amount of acidic glycosaminoglycan (AGAG) than the other organs, and AGAG is believed to bind streptomycin antibiotics and eliminate them passively and slowly. Furthermore, Saito et al. (1971) and one of the present authors (Tachibana et al., 1976) revealed that the AGAG in the lateral wall decreased mark-

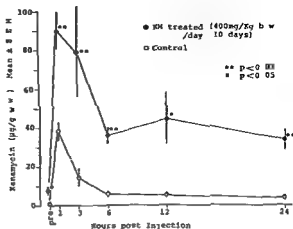


Fig. 8 The time courses of KM levels in the liver. Each value represents the mean and S.E. obtained from 6 samples

edly after successive KM treatment. Therefore, the enhancement of slow KM elimination from the perilymph by successive KM treatment may be caused by the diminished elimination capacity of AGAG in the lateral wall. As for the plexus cochlearis, Mootz et al. (1972) reported on the morphological changes of this structure after KM treatment. Thus, increased and faster accumulation of KM in the pretreated animals may be caused by the increased permeability of KM at this site.

In conclusion, it is plausible that the high accumulation and slow elimination of KM in certain organs and the enhancement of these phenomena by successive treatment are the salient factors in the origin of oto- and nephrotoxicity. The mechanism of the varying susceptibility of each organ to streptomycin antibiotics still remains to be elucidated by modern sophisticated techniques.

ZUSAMMENFASSUNG

Der Verlauf der Kanamycin(KM)konzentrationen in der Perilymphe der Niere, der Leber, dem Gehirn und im Blut wurde durch eine biologische Methode bei normalen Meerschweinchen und bei den mit dauernden KM Injektionen während 10 Tage vorbehandelten Meerschweinchen kalibriert. In der Niere und der Perilymphe der normalen Tiere akkumulierte sich die gegebene KM hoch

gradig und wurde mit einer bedeutenden Verzögerung eliminiert. Diese Tendenz wurde in der Niere und der Perilymphe der vorbehandelten Tiere verstärkt. Die KM im Blut und Gehirn wurde sowohl bei normalen als auch bei vorbehandelten Tieren schnell ausströmen gelassen. Es wird bemerkt, daß die hohe Konzentration der KM in der Perilymphe und der Niere und die späte Ausströmungstendenz und auch das Verstärken dieser Phänomene bei aufeinanderfolgender Vorbehandlung der Tiere sehr wichtig für die Ursache der Oto- und Nephrotoxizität der KM waren.

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A POSSIBLE INVOLVEMENT OF ACIDIC GLYCOSAMINOGLYCANS IN KANAMYCIN OTOTOXICITY

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Abstract The content of acidic glycosaminoglycans (AGAG) was determined quantitatively by electrophoretic microanalysis in the cochlea kidney and brain of the guinea pig. Kanamycin treatment (400 mg/kg body weight/day for 10 successive days) reduced the content of AGAG markedly in the lateral wall of the membranous cochlea. Furthermore, from the results of the ultrastructural and freeze fracturing study, we propose here an excretion system for basic aminoglycoside antibiotics by means of the co-operation of spiral ligament cells and AGAG produced by them. Thus we have tentatively concluded both that kanamycin is excreted via the above mentioned excretion system in the lateral wall of the membranous cochlea and that it damages this system to form the *circulus vitosus* of its ototoxicity.

It is well established that the membranous cochlea contains relatively high amounts of acidic glycosaminoglycans (AGAG, acid mucopolysaccharides) (Saito et al., 1976). The existence of AGAG in the tectorial membrane has attracted great interest in relation to the possible role in the hearing process (Belanger, 1953; Dohlman, 1960; Vilstrup et al., 1961; Tachibana et al., 1973; Yamamichi et al., 1977). However, little is known as to the functional role of AGAG in the lateral wall of the membranous cochlea (LWMC, stria vascularis, spiral prominence + spiral ligament) which shows the highest value among the several tissues in the cochlea (Saito et al., 1971). The present study was designed to clarify one of the functional roles of AGAG in the LWMC in relation to the mechanism of the ototoxicity of basic aminoglycosidic antibiotics.

The improved, rapid quantitation of AGAG by electrophoretic separation, ultrastructural and freeze-fracture methods were

used for the biochemical and morphological analyses of AGAG.

METHODS

Throughout these experiments mature guinea pigs with normal Preyer reflex were used.

(I) Biochemical Analysis

Animals were divided into two groups: the normal control group and kanamycin intoxicated group. Kanamycin intoxication was produced by intramuscular injection of 400 mg of kanamycin sulfate (KM) per kg body weight daily for 10 days continuously. On the day following the last injection, the animals were sacrificed, together with control group. In some animals the threshold of the Preyer reflex was checked daily. The threshold before treatment was used as the zero level for each animal.

More than forty cochleae, as well as several kidneys and brains were kept in 10% neutral formalin at 4°C for 2 weeks to 3 months. The

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Abbreviations used

AGAG: acidic glycosaminoglycans
LWMC: lateral wall of the membranous cochlea
KM: kanamycin (sulfate)
PAM: periodic acid methenamine silver
ChS A, B, C: chondroitin sulfate A, B, C
HA: hyaluronic acid
HS: heparan sulfate
PAPS: 3-phosphoadenosine 5-phosphosulfate

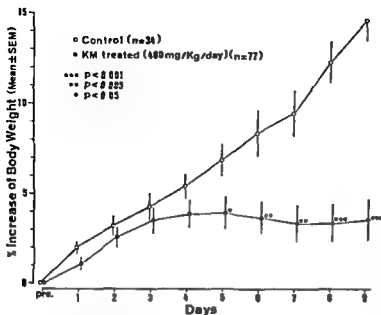
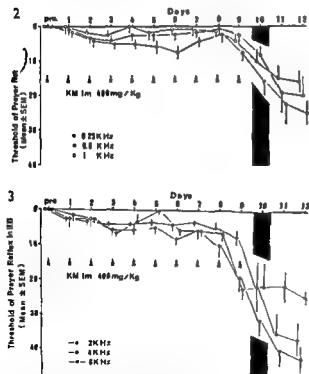


Fig 1 Relative changes of body weight. The percentage increase in the kan (KM) treated group was significantly lower than that in control group after 5 m at the indicated p value. Numbers animals are indicated in the parenthesis.

lateral wall of the cochlea was then dissected and collected in the same solution. The tissues were then cut into small pieces and transferred to absolute acetone for more than 3 days with

several changes of acetone. The samples were dried in the desiccator for more than one week and weighed using an electric balance (minimal detectable change 0.5 μ g). After digi-



Figs 2-3 The shift in the Preyer reflex at the various frequencies. Each value represents the mean of 10 animals. One arrow indicates one injection of KM. In the biochemical study animals were sacrificed after 10 injections (at the day indicated by solid column).

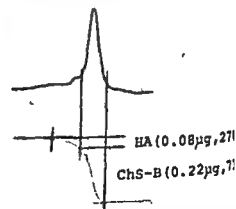
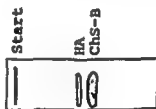


Fig 4 Electrophoretogram on cellulose acetate of the AGAG in the lateral wall of the membranous labyrinth (top). Densitometer tracing (middle) and reading at 600 nm.

Table I Effect of kanamycin (KM) administration on the content of acidic glycosaminoglycans (AGAG)

MC lateral wall of the membranous cochlea

	LWMC	Kidney	Brain
Control	0.74 ± 0.52 (3)	0.09 ± 0.01 (8)	0.08 ± 0.01 (4)
Treated	0.33 ± 0.075 (3)*	0.08 ± 0.01 (8)	0.07 ± 0.01 (4)

* <0.05 compared with control value by Student's *t* test
 AGAG are expressed as weight percent per dry weight. Each value represents the mean ± S.E.M. obtained from the indicated number (in parentheses) of samples.

protein by 5% pronase, AGAG was extracted by ethanol precipitation in the presence of potassium acetate (Meyer et al., 1956; Ito et al., 1970). The extracted AGAG were separated electrophoretically on a cellulose acetate membrane in 0.2 M calcium acetate buffer and stained with 0.5% alcian blue in 3% acetic acid. The content of AGAG was determined by the scanning densitometer. The procedure was essentially the same as reported previously (Saito et al., 1976).

(II) Electronmicroscopic Analysis

(A) Ultrahistochemistry (periodic acid methenamine silver (PAM) impregnation)

The bony wall of the cochlea was opened and fixed in 1% glutaraldehyde and 1% paraformaldehyde in 0.2 M phosphate buffer at pH 4 for 2 hr. After washing in the buffer briefly, the specimens were refixed in 1% OsO₄ for 2 hr, dehydrated in a graded series of alcohol-acetone mixture ending in absolute acetone and embedded in Epon 812 according to the block surface technique. The ultrathin sections obtained were picked up on nylon grids and impregnated according to the method of Yajima (1971). Sections floated on the silver methenamine solution (5% methenamine, 5% silver nitrate, redistilled water, 5% borax solution = 10:1:9:1 in volume) for 10 min at 60°C. After washing with redistilled water, specimens were floated on 0.25% gold chloride for 3 sec and again washed with redistilled water.

(B) Freeze fracturing

The bony wall of the cochlea was opened in solution containing 2.5% glutaraldehyde and 0.1 M phosphate buffer (pH 7.4) and was left in the same fixative for about 4 hrs. For freeze fracturing the specimens were equilibrated with 40% glycerol in saline, then frozen in liquid nitrogen. Platinum carbon replicas were made as reported elsewhere by ourselves (Tachibana et al., 1976, 1976).

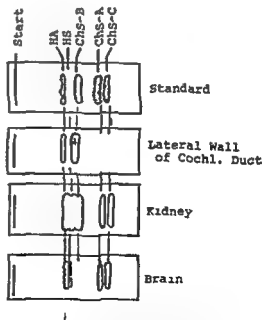
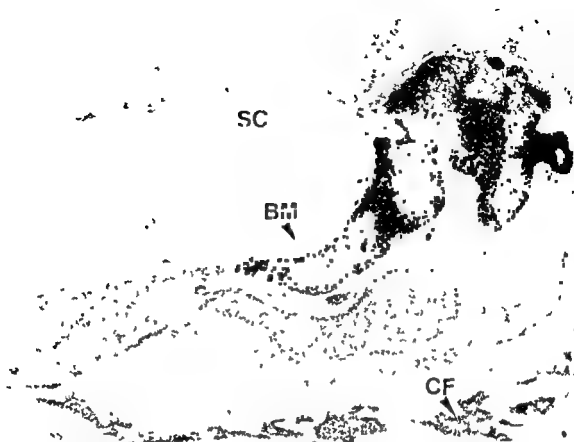


Fig. 5 Electrophoretogram in the tested tissues. The estimation in the kidney was extremely difficult by the tailing of the spots. Also heparan sulfate (HS) was not commercially available as external reference and therefore HS content could not be estimated.



Electronmicrograph of the capillary in the spiral ligament. Periodic acid methenamine silver (PAM) impregnation. Note the impregnated basilar membrane (BM) and the collagen fibres (CF) and the amorphous substance surrounding them. SC spiral ligament cell. Scale $\times 15000$.

RESULTS

The percentage increase in body weight in the two groups is shown in Fig. 1. The value in the KM group was significantly decreased compared with that in control group after 5 days' treatment.

The threshold of Preyer reflex rose abruptly after about 10 injections of KM at all the frequencies examined (Figs 2, 3). In these figures, when the threshold exceeded the maximum sound pressure attainable, a value 5 dB greater than maximum attainable was used.

(I) Biochemical Analysis

The LWMC (lateral wall of the membranous cochlea) contained ChS B (73%) > HA (27%). The kidney contained HA > HS > ChS B > ChS-

C > ChS-A. The brain contained ChS > HA > HS > ChS C. The kinds and patterns did not change after KM treatment in any tissue examined (Figs 4, 5).

The content of AGAG in each tissue is indicated in Table I. Both in the control and the KM treated groups, the LWMC showed the highest content of AGAG among the tissues examined. A significant difference was observed only in the LWMC of the KM control groups by Student's *t*-test (Table I).

(II) Electronmicroscopic Analysis

(A) PAM impregnation

In the LWMC the basement membranes of capillaries were strongly impregnated, particularly those of the spiral ligament (Fig. 1). The collagen fibres between the spiral



Fig. 7 Electronmicrograph of the lateral wall of the membranous cochlea. PAM impregnation. Note the highly impregnated collagen fibres (CF) and the surrounding amorphous

substance between the spiral ligament cell (SC). BC, basal cell of the vascular stria. Arrow, tight junction between them. Scale $1\text{ }\mu\text{m}\times 7800$.

ent cells and the amorphous substance surrounding the fibres were also strongly impregnated (Fig. 6).

Freeze fracturing

Many interesting observations made with this technique will be reported in the near future. In relation to the purpose of this paper, the authors would like to show here the large number of pinocytotic vesicles on the cell membrane of the spiral ligament cells (Fig. 7).

DISCUSSION

In the LWMC (lateral wall of the membranous cochlea) we found PAM impregnated material mainly in the basement membrane of the capillary and collagen fibres and the ground sub-

stance surrounding them between the spiral ligament cells. PAM impregnation is considered to demonstrate primarily the 1,3-glycol and/or alpha-amino groups of glycoproteins and acidic residues of glycosaminoglycans (Rambourg et al. 1967). PAM impregnated material in the LWMC is therefore—at least in part—AGAG. Our ultrahistochemical data show that AGAG exist mainly in the spiral ligament within the LWMC with close correspondence to the previous histochemical data at optical microscopic level (Musebeck et al. 1964; Saito 1967). Considering the evidence that fibroblasts produce AGAG in tissue culture (Dorfman 1970) it seems most likely that AGAG in the LWMC are derived from the spiral ligament cells which are essentially fibroblasts.



Fig. 8. Electronmicrograph of the freeze fractured face of spiral ligament. Note the large number of the pinocytotic vesicles (pv) on the surface of the spiral ligament cell (SC). Also the cross surface of the pinocytotic vesicles (arrows) is seen. CF collagen fibres. Arrow in left corner indicates the direction of the platinum-c shadowing. Scale $1 \mu\text{m} \times 32,500$.

cles (arrows) is seen. CF collagen fibres. Arrow in left corner indicates the direction of the platinum-c shadowing. Scale $1 \mu\text{m} \times 32,500$.

Also Higginbotham (1958) and Mora et al (1959) reported that AGAG or anionic derivatives of synthetic polyglucoses could form complexes with basic aminoglycosides and reduced their toxicities. And the digestion of the drug-AGAG complexes by fibroblasts was suggested. In this connection it is very interesting that spiral ligament cells showed pinocytosis (Fig. 7). Therefore it is likely that the LWMC could have an excretion system for basic aminoglycoside antibiotics by means of co-operation of spiral ligament cells and the AGAG produced from them.

Our present data demonstrated that KM treatment at oto- and nephrotoxic doses decreased the content of AGAG only in the LWMC in contrast to the other organs studied including the kidney. Evidence that KM treat-

ment damages the spiral ligament cells (Tachibana, 1973) and decreases the activity of P-synthase enzyme (one of the key enzymes for AGAG synthesis) in the inner ear (Saito, 1971) is also available. These data demonstrate that the basic aminoglycosides themselves interfere with the proposed excretion system for basic aminoglycosides in the LWMC.

We also found the interesting phenomenon (Tachibana, 1977; Toyoda & Tachibana, 1977) that KM penetrated more rapidly and reached a higher value in the perilymph and excreted more gradually from it in the KM-intoxicated animals than in the control animals.

Taking all of the above data together, we have tentatively concluded that the basic aminoglycosides were excreted to a great degree via the excretion system for basic

cosides in the LWMC and on the other, themselves damaged this system to form *rculus vitosus* for their ototoxicities. Further investigation by more sophisticated methods is necessary to elucidate the putative action system for basic aminoglycosides".
 The inner ear. The difference in mechanism between oto- and nephrotoxicity also remains unanswered. The therapeutic usefulness of G (ChS) against basic aminoglycoside toxicity is already reported (Shida, 1957) and should be re-evaluated.

ACKNOWLEDGEMENT

The authors are grateful to Professor O. Mizukoshi (in Prefectural University of Medicine), Professor P. S. and Professor M. A. Spirtes (in Tulane University School of Medicine) for their continuing encouragement and criticism.

ZUSAMMENFASSUNG

Quantitativen und qualitativen Analysen der sauren Mucopolysaccharide (SGAG) in der Schnecke des Menschen und im Gehirn von Meerschweinchen wurden mit elektrophoretischen Mikroanalysemethoden durchgeführt. Nach Kanamycinbehandlung (täglich 400 mg pro Körpergewicht, 10 Tage) verringerte bedeutend der Gehalt von SGAG der lateralen Wand der häutigen Ecke. Von unseren Untersuchungsergebnissen mit Ultrahistochemie und Gefrierfrakturtechnik wurde ein Exkretionssystem für basische Aminoglykoside vorgeschlagen unter Mitwirkung der Spiralligamenten und der von ihnen produzierten SGAG. Die Möglichkeit, daß Kanamycin von diesem Exkretionssystem abgesondert wird und es deshalb beschädigt, sowie die folgende Ototoxizität in Form eines *rculus vitosus* wurde diskutiert.

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ION TRANSPORT IN GUINEA PIG COCHLEA

I Potassium and Sodium Transport

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Abstract The scala vestibuli and/or tympani in anesthetized guinea pigs was perfused with artificial perilymph containing ^{42}K and ^{22}Na for periods ranging from 5 to 100 minutes. Sound evoked responses were recorded during the perfusion. The activities of ^{42}K and ^{22}Na in perilymph and endolymph were determined with a germanium detector in conjunction with a multichannel analyser. The perfusion of scala vestibuli and/or tympani did not result in appreciable changes in electrical responses. The introduction of ^{42}K and ^{22}Na into the scala tympani resulted in the rapid appearance of these radioisotopes in perilymph of the scala vestibuli. The activities of ^{42}K and ^{22}Na in perilymph of the scala tympani remained low with perfusion of scala vestibuli. This is in agreement with our findings that the clearance of these isotopes was faster in scala tympani than in scala vestibuli. Our results further indicate that the endolymph barrier is impermeable to ^{42}K and extruded ^{22}Na against the concentration gradient. The transport of ^{42}K and ^{22}Na across the cochlear partition was inhibited by anoxia or local application of ouabain. Uptake of ^{42}K in the endolymph from perilymph of the scala vestibuli was comparable to uptake from perilymph of the scala tympani. These results suggest that ^{42}K is actively transported from perilymph to endolymph and ^{22}Na is extruded from endolymph to perilymph and that the active transport of ^{42}K takes place in the stria vasculans. By adopting a two-compartment system the transport rate constant of ^{42}K was computed from our experimental data.

Numerous experiments have shown that the function of the cochlear hair cells is intimately dependent on the unique ion concentration of the endolymph, which is characterized by high potassium and low sodium concentration (Konishi et al, 1966, Konishi & Kelsey, 1968, 1973, Kuypers, 1969). In addition the polarization of the endolymph plays an essential role enabling the hair cells to develop a receptor potential in response to minute acoustic stimuli. The maintenance of the ion concentration in

the endolymph depends upon the structural and functional integrity of the plasma membrane of those cells which serve as the barrier between perilymph and endolymph. It is likely that ototoxic agents can cause destruction of the endolymph-perilymph barrier and consequently lead to a disturbance of mechano-electrical transduction mechanisms in the cochlea.

The permeability of the endolymph-perilymph barrier to various ions can be determined by use of radioisotopes. The concentration of the radioisotopes needed is as a rule smaller than that of the normally existing ions. Therefore one can safely assume that the physicochemical state of the barrier is not altered by the radioisotope tracer. By using radiotracer technique in the cochlea of guinea pig, Rauch et al (1963) argued that Reissner's membrane is a site of active transport of ^{42}K and ^{22}Na . Their work made a significant contribution to understanding the function of Reissner's membrane but their results seem to suggest that their endolymph samples to be contaminated with the perilymph.

This report is the first of a series of proposed experiments using radioisotope methods in an effort to examine the potential alteration of the endolymph-perilymph barrier caused by various ototoxic agents.

This first paper deals principally with transport of ^{42}K and ^{22}Na through the cochlear

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tion under the condition of continuous perfusion in the cochlea of guinea pigs. Our results demonstrate that ^+K is actively transported into the endolymph against an electrochemical gradient and that ^+Na is prevented from entering the endolymph. On the basis of these results, we suggest that the stria vascularis is the principal contributor to active cation transport in the cochlea.

A preliminary report on this work was presented at the 92nd meeting of the Acoustical Society of America (Konishi et al., 1976).

METHODS

Albino pigmented guinea pigs (NIH strain) anesthetized with pentobarbital sodium were used. The cochlear potentials in response to acoustic stimuli were recorded from the basal turn of the cochlea with differential electrodes (Isakaki et al., 1952). The techniques for recording the cochlear potentials, including cochlear microphonics (CM), summating potential (SP), whole nerve action potential of auditory nerve (AP), and endocochlear potential (EP) have been fully described in our previous paper (Konishi et al., 1961). Special care was taken to minimize fluid leakage from the holes prepared for the intracochlear electrodes. In most cases the AP in response to each of eight successive acoustic stimuli was averaged by a computer. The tone bursts used

effectively

The perfusion technique employed was similar to that described in our previous paper (Konishi & Kelsey, 1968). When both scala vestibuli and tympani were perfused, a perfusion pipette whose tip was approx. 100 μm in diameter was inserted into the scala tympani at the basal turn of the cochlea and a hole to serve as an outlet was made in the scala vestibuli of the basal turn. When the scala vestibuli

or scala tympani was perfused separately, a perfusion pipette was placed in the corresponding scala of the basal turn and an outlet was made at the apex. The perfusion rate was approximately 2 $\mu l/min$ and the period of perfusion ranged from 5 to 60 min.

^{42}K (12.7 hours half-life) of high specific activity and ^{22}Na (2.6 years half-life) were obtained in chloride form from New England Nuclear, Boston, Massachusetts. These original radioactive solutions were mixed with modified Ringer's solution so that the perfusate had the following composition (mM): NaCl 137, KCl 5, $CaCl_2$ 2, NaH_2PO_4 1, $MgCl_2$ 1, $NaHCO_3$ 12, glucose 11. As a rule the activity of ^{22}Na in the perfusate was 125 nCi/ μl and that of ^{42}K ranged from 250 to 300 nCi/ μl at the time of preparation. The perfusate was kept at room temperature (18°C).

The technique of sample collection of the endolymph has been described previously (Mandelsohn & Konishi, 1969) and therefore only a brief description will be given here. A double-barrelled micropipette was prepared whose tip was approx. 5–10 μm in diameter. The tip of one barrel used to collect the endolymph was then filled with water equilibrated mineral oil. The other barrel was filled with Ringer's and used as an EP recording electrode. The double barrelled pipette, mounted on a micromanipulator, was inserted into the scala media of the basal turn through the spiral ligament. About 1 μl of fluid was collected by gentle suction when the potential recording barrel registered stable EP, indicating the tip to be in the scala media. The mineral oil prevented fluid from entering the barrel until suction was applied. When the endolymph was collected smoothly, EP did not show any significant change. When EP showed a sudden drop or decreased continuously by more than 10 mV, the sample was discarded.

Samples of perilymph were taken from the scala vestibuli and scala tympani of the basal turn of the cochlea. A single glass micropipette was used whose tip was ca. 100 μm in diameter. The shank was coated with lacquer so as

to form a small bead at about 100 μm from the tip. The tip was then filled with water-equilibrated mineral oil. After the tips of the differential electrodes were temporarily lifted from the cochlea, the collection micropipette was inserted into either scala vestibuli or tympani. About 1 μl of perilymph was obtained by applying gentle suction. The whole procedure of collection of endolymph and perilymph required 3 to 4 min. Samples were taken at times ranging from 5 to 60 min after the start of perfusion. Separate animals were used for each time, since it is difficult to collect more than one sample of the endolymph and perilymph in the scala vestibuli and tympani. An exception to the above was made in one set of experiments in which the radioactivity in the perilymph was determined as a function of time with the same animal.

The activity of ^{42}K and ^{22}Na was determined using a solid state germanium detector in conjunction with a multichannel analyser. The system was calibrated with known quantities

^{42}K and ^{22}Na by determining the total number of counts in selected channels for a live time of 1000 sec. This time was sufficient to reduce the standard deviation in the sample from counting errors to less than 5% of the total counts. Activity determinations were corrected for the decay of ^{42}K . The samples containing ^{42}K and ^{22}Na were transferred into 10 μl calibrated glass pipettes. Both ends of the samples were sealed with water-equilibrated mineral oil. The volumes of samples were calculated by measuring their length under a microscope. Each sample in a 10 μl calibrated pipette was carefully centred under the detector crystal in a specially constructed holder. Under the condition used, small changes in the position of the sample did not cause detectable changes in the geometrical efficiency. Thus the method used allowed us to express the activities of ^{42}K and ^{22}Na in terms of $\text{nCi}/\mu\text{l}$.

Special methods and procedures used in specific experiments will be described in the Results section.

RESULTS

The results reported here were based on obtained from 110 guinea pigs.

1 Cochlear potentials during perfusion

The average magnitude of CM in response to 6 kHz tone bursts at 70 dB SPL was 450 μV when the CM was recorded from the bulla. The CM was stable during perfusion of the scala vestibuli and tympani when it was taken to remove the accumulated fluid from the auditory bulla. In a few cases the CM was slightly increased in magnitude by a factor of 10% during the first few minutes of perfusion. There was a slight decrement of CM during perfusion, ranging from 5 to 20 minutes. In cases of a period of perfusion longer than 20 minutes, the CM showed a gradual decrease in magnitude but the decrease of CM did not exceed more than 30% of the initial CM. When the perfusion was carried out in the scala vestibuli or tympani separately, the suppression of CM was less in both cases than when the entire perilymphatic space was perfused.

The magnitude of AP to 6 kHz tone bursts at 70 dB SPL showed a larger variation than that of the CM. It ranged from 250 to 1000 μV with a mean value of 180 μV . The AP was temporarily suppressed during the first 2-5 minutes of the perfusion. The decrease in AP ranged from 10 to 50% of the original value. The AP generally showed full recovery 10 minutes after the perfusion was initiated. The AP was stable during the rest of the perfusion period. When the scala tympani was perfused separately, the temporal decrease in AP associated with initiation of perfusion was similar to that observed during the perfusion of the entire perilymphatic space. The decrease in AP was much less in the perfusion of the scala vestibuli than in that of the scala tympani.

Changes in SP were noted during the first few minutes of perfusion. When the perilymphate was introduced into the scala vestibuli, the negative SP was increased in magnitude. The perfusion of the scala tympani tended

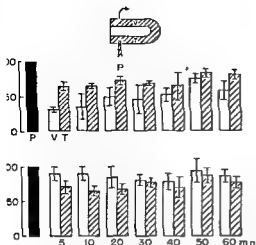


Fig. 1. Mean activities of ^{41}K (upper panel) and ^{22}Na (lower panel) in samples obtained from scala vestibuli (V) and from scala tympani (T) at various durations of perfusion indicated below the bars. The perilymphatic space was perfused as shown in the top diagram. The activities are normalized by taking those in the perfusate (P) as 100%. Four to six animals were used for each perfusion time, except for the 5 min perfusion in which only 3 animals were used. Vertical range bars indicate standard deviations.

like the SP less negative and sometimes a relative SP was observed. However, such changes in SP gradually disappeared 10 min after perfusion was initiated and the SP was stable during the rest of the perfusion period in the preparations which were used for determination of activity in the cochlear fluids.

EP was not recorded continuously during perfusion but was measured at the time of collection of the endolymph. The average value of the EP was 82.5 mV, which was comparable to that obtained from non-perfused cochlea. During collection of the endolymph, the EP did not show a decrease of more than 10 mV. The CM and especially the AP were suppressed but decreases in these responses were less than 20% when the endolymph was collected.

The perilymph were taken within 10 seconds. The decrease in the CM and AP ranged from 20 to 50%. However, in most cases the

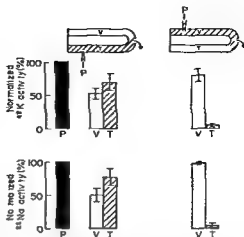


Fig. 2. Mean activities of ^{41}K (upper panel) and ^{22}Na (lower panel) under two different perfusion conditions as shown in the top diagrams. The duration of perfusion was 20 min. Activities in perilymph of scala vestibuli (V) (□) and scala tympani (T) (■) are normalized by taking those in perfusate (P) (■) as 100%. Six animals were used for each condition. Vertical range bars indicate standard deviations.

CM and AP showed partial recovery after collection was completed.

2 Concentration of ^{41}K and ^{22}Na in perilymph

When the perfusate containing ^{41}K and ^{22}Na was introduced into the scala tympani of the basal turn and both scala vestibuli and tympani were perfused, the concentration of ^{41}K and ^{22}Na in samples of the perilymph taken from the scala tympani ranged from 65 to 85% of those in the perfusate and remained almost unchanged as the period of perfusion was prolonged (Fig. 1). ^{41}K and ^{22}Na in samples from the perilymph of the scala vestibuli were detectable 5 minutes after perfusion was initiated. As the period of perfusion was prolonged, the concentration of ^{22}Na in the perilymph of the scala vestibuli did not increase but it was always higher than that in the perilymph taken from the scala tympani. On the other hand, the concentration of ^{41}K in the perilymph of the scala vestibuli had a tendency to increase as the period of perfusion increased and it was noted that samples from the scala vestibuli in-

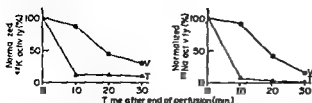


Fig 3 One example of clearance of ^{42}K (left panel) and ^{22}Na (right panel) after the end of perfusion of perilymphatic space. ● Activity in perilymph of scala vestibuli, ▲ activity in perilymph of scala tympani, T The activities are normalized by expressing those obtained at end of perfusion as 100%. Abscissa Time elapsed after perfusion. Three animals were tested and 2 other animals showed similar clearance.

indicated a substantially lower concentration of ^{42}K than those obtained from the scala tympani.

It is seen in Fig 2 that when the scala tympani alone was perfused for a period of 20 minutes, concentrations of ^{22}Na and ^{42}K in samples taken from the scala vestibuli were about 30% lower than those obtained from the scala tympani. Concentrations of ^{42}K and ^{22}Na in the perilymph of the scala vestibuli after perfusion of the scala vestibuli alone showed 70 to 90% and 90 to 100% respectively of those in the perfusate. Surprisingly the activity of ^{22}Na and ^{42}K in samples taken from the scala tympani showed only a few percent of the activity in the perfusate.

We examined the activity of ^{22}Na and ^{42}K in the perilymph of both scala vestibuli and tympani when the scala vestibuli was perfused for a period of 20 min in animals in which the cochlear aqueduct had been surgically obstructed. Obstruction of the cochlear aqueduct was performed by drilling with a fine burr across the cochlear aqueduct. A minute amount of bone wax was applied to obstruct the cochlear aqueduct. In those animals CM and AP were not suppressed after the obstruction. The activities of ^{22}Na and ^{42}K in samples obtained from the scala tympani increased by a factor of 2 to 3 but remained lower than those in samples from the scala vestibuli.

The activities of ^{22}Na and ^{42}K in samples taken from the perilymphatic space decreased

rapidly when the perfusion was discontinued. Fig 3 shows clearance of ^{22}Na and ^{42}K at the end of perfusion of the scala vestibuli and tympani in one animal in which samples of perilymph were periodically taken. The decline in activity was much faster in samples obtained from the scala tympani than in those from the scala vestibuli. Thirty minutes after the end of perfusion the loss of activity of ^{42}K was about 80% in the perilymph of the vestibuli, whereas ^{22}Na was scarcely detectable in the perilymph of the scala tympani. There was no noticeable difference in the rate of clearance between ^{22}Na and ^{42}K .

3 Concentration of ^{42}K and ^{22}Na in endolymph

1 Normal guinea pigs It is noted in Fig 1 that when the scala vestibuli and tympani were perfused, the ^{42}K concentration in the endolymph exceeded that in the perilymph in the scala tympani within 5 min after perfusion.

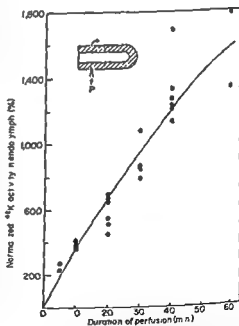
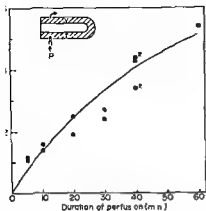


Fig 4 Uptake of ^{42}K in endolymph as a function of perfusion duration. The scala vestibuli (V) and tympani (T) were perfused as shown in inset diagram at top. Activity in endolymph is normalized by taking that obtained at end of perfusion as 100%. ● Data points. The line is the least squares fit to the points (see text).



5 Uptake of ^{22}Na in the endolymph as a function of perfusion duration. See caption to Fig. 4

commenced. As perfusion continued to 40 min, the concentration of ^{42}K rose to about 12% of the concentration of ^{42}K in samples taken from the scala tympani. As shown in Fig. 5, the concentration of ^{22}Na in the endolymph was less than 1% of that in samples obtained from the scala tympani. Even when perfusion was prolonged by 60 minutes, the ^{22}Na concentration in the endolymph did not reach 5% of that found in the perilymph from the scala tympani.

As noted above, in the perfusion of the scala tympani alone, the activities of ^{42}K and ^{22}Na in the scala vestibuli were comparable to those in the scala tympani. When the site of perfusion is switched from scala tympani to scala vestibuli, their activities in the scala tympani were less than those in the scala vestibuli. However, the activities of ^{42}K and ^{22}Na in the endolymph did not show any marked differences under these two conditions, when their activities were normalized by expressing those obtained from the perilymphatic space into which the perfusate was introduced as 100%.

These findings were further substantiated by a dual perfusion. The simultaneous perfusions were carried out with 'hot' solution containing ^{42}K and ^{22}Na and 'cold' solution without ^{42}K and ^{22}Na . Three experimental procedures were employed. Fig. 6 shows the activities of ^{42}K and ^{22}Na in the perilymph and

endolymph under these conditions of perfusion for a period of 20 min. In the first procedure "hot" solution was introduced into both scala vestibuli and tympani (left panel in Fig. 6). In the second procedure the "hot" solution was introduced into the scala tympani and "cold" solution was simultaneously introduced into the scala vestibuli (middle panel). In the third procedure a similar perfusion was carried out in the reverse manner, "hot" perfusion of scala vestibuli and "cold" perfusion of scala tympani. The electric responses during the dual perfusion did not show differences from those observed in the perfusion of the scala tympani. The activities of ^{42}K and ^{22}Na were, of course, very weak in the samples taken from the scala which was perfused with the "cold" solution. On the other hand, their activities in the samples taken from the scala into which the "hot" perfusate was introduced, were comparable to those found in the "hot" perfusate. The activities of ^{42}K and ^{22}Na in the

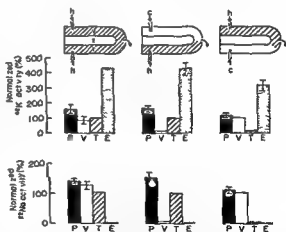


Fig. 6 Mean uptake of ^{42}K (upper panel) and ^{22}Na (lower panel) in endolymph *E* and perilymph of scala vestibuli *V* and scala tympani *T* under three different perfusion conditions as shown in diagrams at top left: perfusion of scala vestibuli and tympani with hot perfusate *h* and middle simultaneous perfusion of scala tympani with "hot" perfusate and perfusion of scala vestibuli with cold perfusate *c*; right simultaneous perfusion of scala vestibuli with "cold" perfusate and perfusion of scala tympani with hot perfusate. Duration of perfusion was 20 minutes. Activities are normalized to those of *V* or *T*. Four animals were used for each condition. Vertical range bars indicate standard deviations.

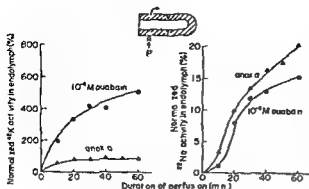


Fig. 7 Effects of anoxia (\blacktriangle) and 10^{-4} M ouabain (\bullet) on uptake of ^{42}K (left panel) and ^{22}Na (right panel) in endolymph. Scala vestibuli and tympani were perfused as shown in top diagram. Ordinate: Activity in endolymph normalized to that in perilymph of the scala tympani. Two to three animals were used to obtain each data point.

endolymph showed little difference under these three perfusion conditions.

2 Effect of ouabain The effect of ouabain on the uptake of ^{42}K and ^{22}Na in the endolymph was examined by adding ouabain to the perfusate. The concentration of ouabain was 10^{-4} M. Introduction of ouabain into the perilymphatic space resulted in the suppression of cochlear potentials to acoustic stimuli. The decline of the electric responses we observed was similar to that reported previously (Konishi & Mendelsohn, 1970). As demonstrated in Fig. 7, the concentration of ^{42}K in the endolymph was diminished by 10^{-4} M of ouabain in the perfusate. This suppressive effect was somewhat greater when the concentration of ouabain was increased to 10^{-3} M.

The concentration of ^{22}Na in the endolymph was increased by local application of ouabain (Fig. 7). Compared with that found in normal animals (Fig. 5), ^{22}Na concentration increased slightly 10 min after the start of perfusion and as the perfusion continued the rate of increase of ^{22}Na concentration increased. With 10^{-3} M of ouabain the ^{22}Na concentration in the endolymph was greater than that obtained with 10^{-4} M.

3 Effect of anoxia The cochlear responses

to acoustic stimuli showed a rapid decline after the intravenous injection of a lethal dose of pentobarbital sodium. The perfusion of the scala vestibuli and tympani was carried out after the injection. The concentration of ^{22}Na in the endolymph did not reach the level of concentration in the scala tympani (Fig. 7) in contrast to that shown in Figure 4. The concentration of ^{22}Na in the endolymph as shown in Figure 7, increased in the post-anoxic state. As the duration of perfusion increased, the activity of ^{22}Na increased markedly. After 60 min of perfusion reached 20% activity in the perilymph of the scala tympani.

4 Effect of perfusion In order to ascertain that the perfusion did not alter the permeability of the cochlear partition to ^{42}K or ^{22}Na , perfusion of the perilymphatic space was performed with 'cold' perfusate for a duration ranging from 20 to 60 min. Immediately after the initial perfusion, perfusate containing ^{42}K and ^{22}Na was again introduced into the perilymphatic space. The activities of ^{42}K and ^{22}Na in the endolymph did not show any differences from those obtained without 'cold' perfusion which were described in the previous section.

4 Quantitative aspects of ^{42}K and ^{22}Na transport across the cochlear partition

In order to simplify the treatment of the transport process occurring in the guinea pig cochlea, assume that the cochlea can be represented by two compartments. Let the inner compartment represent the scala media with the endolymph and the outer compartment, the perilymphatic space filled with perilymph. Let J_{in} (mmole/min) denote the net flux of given nonradioactive ions from the outer compartment to the inner compartment and their flux in the opposite direction by J_{out} . The corresponding flux of radioactive ions may be denoted by $^*J_{in}$ and $^*J_{out}$. Under perfusion conditions, we may assume that the concentration of ions in the perilymphatic space is constant. For this condition the two

t system in effect becomes a one compartment system. The unidirectional fluxes of radioactive ions can be expressed by

$$J_{in} = \frac{C_{out}}{C_{in}} J \quad \text{and} \quad J_{out} = \frac{C_{in}}{C_{out}} J \quad (1)$$

where C_{out} and C_{in} are concentrations of non-radioactive ions in the outer and inner compartment respectively, and $*C_{out}$ and $*C_{in}$ are concentrations of radioactive ions in the outer and inner compartment respectively. In the steady state, the net flux of nonradioactive ions is zero. Thus

$$J_{in} = J_{out} \quad (2)$$

From eqs (1) and (2)

$$\begin{aligned} J_{in} &= \frac{C_{out}}{C_{in}} J \\ J_{out} &= \frac{C_{in}}{C_{out}} J \end{aligned} \quad (3)$$

The rate of change in the concentration of radioactive ions in the inner compartment can be expressed by

$$V_{in} \frac{d*C_{in}}{dt} = *J_{in} - *J_{out} \quad (4)$$

where V_{in} is the volume of the inner compartment.

From eqs (3) and (4)

$$V_{in} \frac{d*C_{in}}{dt} + J \frac{*C_{in}}{C_{in}} = J \frac{*C_{out}}{C_{out}} \quad (5)$$

Since the perilymphatic space is being perfused, $*C_{out}$ is assumed to be constant. By solving eq (5)

$$C_{in} \exp[Jt/V_{in} C_{in}] = \frac{C_{in}}{C_{out}} *C_{out} \exp[Jt/V_{in} C_{in}] + \text{const} \quad (6)$$

When $t=0$, $*C_{in}=0$

$$\text{thus } \text{Const} = \frac{-C_{in} *C_{out}}{C_{out}} \quad (7)$$

From eqs (6) and (7),

$$\frac{*C_{in}}{*C_{out}} = \frac{C_{in}}{C_{out}} (1 - \exp[-Jt/V_{in} C_{in}])$$

or

$$\ln\left(\frac{C_{in}}{C_{out}} \frac{*C_{in}}{*C_{out}}\right) = \ln\left(\frac{C_{in}}{C_{out}}\right) - \frac{J}{V_{in} C_{in}} t \quad (8)$$

From eq (8) the transport rate constant, $J/V_{in} C_{in}$ can be calculated, if C_{in} , C_{out} , $*C_{in}$ and $*C_{out}$ are known.

The concentration of $*K$ in the endolymph reported by various investigators (in guinea pigs Johnstone et al, 1963, Konishi & Kelsey, 1973, in rats Boshier et al, 1973) ranged from 130 to 190 mM. Assuming that C_{in} of $*K$ is 150 mM and C_{out} of $*K$ is 5 mM, Fig. 8 shows the relationship between $\ln(C_{in}/C_{out} *C_{in}/*C_{out})$ and t , perfusion time, expressed by eq (8). By utilizing the least square best fit, the regression line can be expressed by

$$\ln\left(\frac{C_{in}}{C_{out}} \frac{*C_{in}}{*C_{out}}\right) = 3.40 - 0.0126t \quad (9)$$

Using an analysis of variance technique (Dixon & Massey, 1957), the data were tested for linearity and found not to differ significantly ($P=0.05$) from the above line. The transport rate constant for $*K$ was 0.0126 min^{-1} and the half-time for exchange was 55.1 min.

The concentration of $*Na$ in the endolymph determined by various investigators (Johnstone et al, 1963, Konishi & Kelsey, 1973, Boshier et al, 1973) shows considerable variations and ranges from 2.5 to 0.5 mM. Assuming that the concentration of $*Na$ is 1 mM in the endolymph and 140 mM in the perilymph, the transport rate constant was 0.021 min^{-1} and the half time exchange was 32.6 minutes. If the concentration of $*Na$ is 2 mM in the endolymph, the transport rate constant becomes 0.007 min^{-1} and the half time exchange 94.8 min. Since the flux (J) is the product of the volume, concentration and rate constant, we may determine the ratio of the flux of $*K$ to that of $*Na$. When the $*Na$ concentration is assumed to be 1 mM or 2 mM, the flux ratios are 90 and 135 respectively.

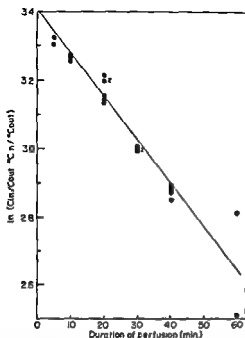


Fig. 8 $\ln \{(C_{in}/C_{out}) - (*C_{in}/*C_{out})\}$ as a function of duration of perfusion. C_{in} and C_{out} represent nonradioactive K concentration in endolymph and perilymph (assumed to be 150 mM and 5 mM respectively). $*C_{in}$ and $*C_{out}$ represent radioactive K concentration in endolymph and perilymph respectively. The regression line obtained by squares best fit has a slope of 0.013, an intercept of correlation coefficient is 0.92.

DISCUSSION

Since Smith et al. (1954) demonstrated that the endolymph of guinea pig has a very high concentration of 4K and a very low concentration of 4Na , extensive studies of the distribution of 4K and 4Na in the cochlear fluids have been carried out in various species (Katsuki, 1965) and under various pathological conditions (Mendelsohn & Katzenberg, 1972; Bosher et al., 1973; Cohen et al., 1971). The movement of 4K and 4Na across the cochlear partition is very important to revealing the basic mechanism of cochlear function and yet only a few studies have been reported (Rauch et al., 1963; Choo & Tabowitz, 1964, 1965). In reviewing their reports critically, their results and interpretations are inconsistent and conflicting. It is likely that these conflicting results can be attributed to technical difficulties in the collection of endolymph without contamination by

the perilymph and secondly to a lack of adequate monitoring of the cochlear fluid during the introduction of radioisotopes in cochlear fluids. In our experiments, evoked responses were periodically recorded during the perfusion and the EP was monitored during collection of the endolymph. The endolymph collected was discarded if it decreased its magnitude more than 10 mV during collection. The determination of 4Na in the endolymph samples collected by this method (Konishi & Kelsey, 1973) is in agreement with the report by Bosher et al. (1973) or Johnstone et al. (1963). It is reasonable to assume that contamination of the perilymph is negligible in our experiments.

In addition, there is a good possibility of chemical injuries of the cochlear partition associated with the intracochlear injection of ^{42}K for the conflicting results reported previously (Rauch et al., 1963). It has been suggested that the electrical responses of the cochlea are modified by either alteration of the electrolyte concentration of the perilymph (Konishi & Kelsey, 1968, 1970, 1973) or changes in hydrostatic pressure in the cochlear fluids (Tasaki et al., 1954). Therefore, these changes may cause modification in the permeability of the cochlear partition to electrolytes. For example, isotonic KCl solutions used by Rauch et al. (1963) may have resulted in chemical injury to the cells of Reissner's membrane. Our experiments show that the sound evoked response did not change substantially during the perfusion of the perilymphatic space. It is likely, therefore, that any changes in hydrostatic pressure caused by perfusion and replacement of perilymph with the perfusate used by us were not of sufficient magnitude to disturb cochlear responses. This conclusion was further substantiated by the fact that uptake of ^{42}K and ^{22}Na in the endolymph was not modified by prior perfusion of the perilymphatic space with 'cold' solution.

The activities of ^{42}K and ^{22}Na in the perilymph of the scala vestibuli of the basal turn are detectable within 5 min after the f

te is introduced into the scala tympani of basal turn (Fig. 1). The presence of the activity indicates that the perfusate reaches the scala vestibuli not only through the helicotrema (longitudinal pathway), but also through the radial pathway. It is unlikely that ^{42}K and ^{22}Na introduced into the scala tympani diffuse through the scala media and then reach the scala vestibuli, as Jahnke (1975) reported that zonular occludens of non sensory epithelia seal cochlear duct. Thus the spiral ligament serves principally as a radial pathway, in agreement with a previous report in which Lindorf et al. (1962) observed the rapid fluid change in the spiral ligament by using the ^{51}Cr staining technique. Rauch et al. (1963) injected isotonic ^{42}K or $^{22}\text{NaCl}$ solution into the scala vestibuli and measured uptake of ^{42}K and ^{22}Na in the endolymph in order to determine transport occurs across Reissner's membrane. They interpreted their results as showing that radioisotopes in the endolymph are transported across Reissner's membrane. However, our findings indicate that ^{42}KCl or $^{22}\text{NaCl}$ solution injected into the scala vestibuli easily diffuse into the spiral ligament and therefore do not support their interpretation. One puzzling result is the fact that perfusion of the scala vestibuli with radioactive solution results in low activities in perilymph of the scala tympani and it occurs consistently regardless of the duration of perfusion. This result can be explained by the rapid clearance of radioisotopes in the scala tympani. As shown in Fig. 3 the clearance of ^{42}K and ^{22}Na is much faster in the scala tympani than in the scala vestibuli. The perfusate introduced into the scala vestibuli reaches the scala tympani through the spiral ligament but the clearance of ^{42}K and ^{22}Na is so fast in the scala tympani that an increase in concentration of these radioisotopes cannot be observed. This may account for the fact that an increase in K concentration in the perilymph of the scala tympani is more effective in suppressing the CM and AP than that of the scala vestibuli (Tasaki & Fernandez 1952). Another possible explanation is

that as the scala tympani communicates the subarachnoidal space through the cochlear aqueduct, opening of an outlet in the scala tympani may create leakage of the cerebrospinal fluid into the scala tympani and, as a consequence, the concentration of these radioisotopes in the scala tympani is diluted with the cerebrospinal fluid. This possibility can not be a major factor as obstruction of the cochlear aqueduct did not substantially increase the activities of ^{42}K and ^{22}Na in the scala tympani. Kimura & Perlman (1956) reported that profound changes in the inner ear are produced only when the inferior cochlear vein is obstructed at the level at which some of the collaterals are also obstructed. From the fact that the surgical blocking of the cochlear aqueduct did not suppress the electrical responses of the cochlea, it is apparent that the surgical procedure used to obstruct the cochlear aqueduct did not interfere with the venous drainage of the cochlea.

We have consistently found that, when the perilymphatic space was perfused with artificial perilymph containing ^{42}K , the endolymph took up ^{42}K very quickly and within 5 min after perfusion, the ^{42}K concentration in the endolymph was greater than in the perilymph (Fig. 4). On the other hand, the uptake of ^{22}Na in the endolymph was extremely small (Fig. 5). Although our experimental conditions are different from those used by Rauch et al. (1963), our results are qualitatively in agreement with their findings. Because the endolymph is a rich ^{42}K and low ^{22}Na medium and positively polarized with respect to the perilymph, the transport of ^{42}K ions is against the electrochemical gradients. Also the transport of ^{42}K and ^{22}Na was inhibited by anoxia and ouabain as shown in Fig. 7. Ouabain generally inhibits active transport (Caldwell & Keynes, 1959) and it is reasonable to conclude that the uptake of ^{42}K across the cochlear partition is energy dependent.

According to Ullrich (1974), the net flux ΔJ , can be expressed by the following equation

$$\Delta J = J_{\text{solvent drag}} + J_{\text{diffusion}} + J_{\text{active}}$$

In the steady state, $\Delta J = 0$

$$\text{Therefore } -J_{\text{active}} = J_{\text{solvent drag}} + J_{\text{diffusion}}$$

The ^+K leakage flux from the endolymph to perilymph can be assumed to be either flux caused by diffusion or a combination of diffusion and solvent drag. Presumably ^+Na flux from the perilymph to endolymph is passive. These interpretations are quite in agreement with the findings that anoxia or local application of ouabain results in an increase in the concentration of ^+Na and a decrease of ^+K concentration in the endolymph (Mendelsohn & Konishi, 1969; Konishi & Mendelsohn, 1970).

It is difficult to determine the location of the active transport, because of the morphological heterogeneity of the cochlear partition. However, it is evident from the results obtained by the simultaneous perfusion of the scala vestibuli (or tympani) with "cold" solution and perfusion of the scala tympani (or vestibuli) with "hot" solution that the lateral wall of the cochlear partition is responsible for active uptake of ^{42}K and extrusion of ^{22}Na and that Reissner's membrane does not play a major role in the active ion transport. Jahnke (1975) reported on the fine structure of the freeze-fractured intercellular junction in the cochlea of guinea pigs and found that the intercellular space of the stria vascularis is sealed to the spiral ligament by a very tight junction of basal cells. Kuypers (1969) reported that the highest activity of Na - K activated ATPase was found in the stria vascularis of the guinea pig. The oxygen consumption of freshly dissected stria vascularis is high too, according to Chou & Hughes (1964). In conjunction with these findings, our results suggest that the stria vascularis is a site of active transport. The marginal cells are the only cells in the stria vascularis that are contiguous with the endolymph and characterized by basal infoldings and large dense mitochondria. These are characteristic for cells of other organs involved in ion trans-

port (Pease, 1956). It is likely that the cells may play an important role in the transport of ^+K and ^+Na .

Compartmental analysis has proved when applied to problems pertaining to electrolytes and water movement in the cochlea (Llaurado, 1973). Recently Mnich (1975) applied compartmental analysis and estimated the efflux of ^{24}Na in isolated cochlea from a brane section containing the stria vascularis into artificial endolymph. The compartmental model must be consistent with what is known about the physiological configuration of the system under consideration. The endolymph is absorbed by certain tissues along the cochlear partition, even though the question of the site of absorption remains unanswered. In this respect, a three-compartment system nearly represents the physiological configuration of the cochlear fluids than does the two-compartment model used in this study. In the three-compartment model, the compartments are identified with the endolymph, the perilymphatic space as previously described. The third compartment is identified with the tissues which absorb the endolymph. This third compartment is considered to be open, since vascularization of that compartment may result in the transport of ions out of the regions of interest. An open three-compartment system is used in modelling the transport of ^{42}K from the perilymph to the endolymph. It is found that the uptake of ^{42}K is represented by the sum of two exponentials. Our results show that the hypothesis that ^{42}K uptake in the endolymph is represented by a single exponential cannot statistically be rejected at the $p=0.05$ level. Therefore the absorption of the endolymph does not seem to be a dominant factor in the experiments and the closed two-compartment model can be used to reproduce the kinetics of the uptake of ^{42}K in the cochlear fluid.

The transport rate constant obtained from compartmental analysis is based on the assumption that the concentration of ^{42}K in the stria vascularis is of the same order of magnitude as that in the scala tympani. Presumably

former is slightly higher than the latter and actual transport rate constant for K may slightly from that calculated. As noted in (8), a slight difference in C_m caused a large variation in the transport rate constant. Thus, termination of the transport rate constant ^{41}K requires a precise value of the concentration of ^{41}K in the endolymph. It is difficult to estimate the transport rate constant for ^{41}K using data available in literature. A more accurate determination of ^{41}K concentration with sensitive equipment is needed.

The dynamic equilibrium of electrolytes in cochlear fluids is maintained by diffusion, osmosis, bulk movement owing to hydrostatic pressure and active transport. The results described here demonstrate our first step to elucidate the mechanism of electrolyte exchange between perilymph and endolymph while maintaining a concentration gradient across the endolymph-perilymph barrier. Our results illustrate, contrary to findings by Much et al. (1963) that the stria vascularis is the site of active ^{41}K transport into the endolymph and ^{23}Na extrusion. The cochlear partition is permeable to both ^{41}K and ^{23}Na . An understanding of the mechanism controlling homeostasis in the cochlear fluids is essential for studying the pathogenesis of various hearing impairments which may occur as a result of a disturbance of the dynamic equilibrium of electrolytes in the cochlear fluids.

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ZUSAMMENFASSUNG

Die Scala vestibuli und/oder tympani von betäubten Meerschweinchen wurden mit künstlichem Perilymph, welches ^{41}K und ^{23}Na enthält, für Zeitdauern von 5 bis 60 Minuten behandelt. Die während der Behandlung durch die Membran hervorgerufenen Reaktionen wurden protokolliert. Die Perilymph und Endolymph enthaltenen Aktivitäten wurden mit Hilfe eines Germaniumdetektors und eines

Mehrkanalanalysators bestimmt. Die Behandlung der Scala vestibuli und/oder tympani hatte keine nennenswerten Veränderungen der elektrischen Reaktionen zur Folge. Die Eingabe von ^{41}K und ^{23}Na in das Perilymph der Scala tympani verursachte das fast sofortige Erscheinen dieser Radioisotopen im Perilymph der Scala vestibuli. Die ^{41}K und ^{23}Na Aktivitäten im Perilymph der Scala tympani verblieben niedrig beim Behandeln der Scala vestibuli. Dies stimmt mit unserer Entdeckung überein, daß das Abklingen dieser Radioisotopen in der Scala tympani schneller vor sich ging als in der Scala vestibuli. Unsere Resultate zeigen außerdem, daß sich das Endolymph mit ^{41}K anreicherte, während es ^{23}Na abschied und zwar gegen die Konzentrationsgradienten. Der Transport von ^{41}K und ^{23}Na durch die Cochlea Trennwand wurde durch Anoxie oder durch lokale Anwendung von Ouabain verhindert. Das Anreichern von ^{41}K im Endolymph vom Perilymph der Scala vestibuli war vergleichbar mit dem Anreichern vom Perilymph der Scala tympani. Diese Resultate deuten darauf hin, daß ^{41}K aktiv vom Perilymph zum Endolymph transportiert und ^{23}Na vom Endolymph zum Perilymph abgeschieden wird und daß der aktive Transport von ^{41}K in der Stria vascularis stattfindet. Die Transportgeschwindigkeitskonstante von ^{41}K wurde von unseren experimentellen Werten mit Hilfe eines angenommenen Zweizellensystems berechnet.

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GENTAMICIN-INDUCED MITOCHONDRIAL DAMAGE IN INNER EAR SENSORY CELLS OF THE LIZARD *CALOTES VERSICOLOR*

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Abstract Earlier morphological and histochemical studies of the effects on inner ear sensory cells caused by aminoglycoside antibiotics have failed to give sufficient information of the genesis of these effects. The present study, focused on the phases of progressive mitochondrial changes in sensory cells of the lizard basilar papilla, induced by consecutive large doses of gentamicin. The mitochondria react by swelling, changes in the configuration and number of the cristae and formation of matrix inclusions. Myelin figures are a consistent finding in degenerating cells after gentamicin exposure. These are shown to be derived from changed mitochondria. The final product is an onion-like structure which is built of multiple membranes. There is a marked difference in reaction to the damage between individual mitochondria in the same cell. This difference is also evident between individual sensory cells in the same specimen. By studying the phases of the mitochondrial breakdown process in the sensory cell, some additional information on the changes in cell metabolism caused by ototoxic drugs may be extracted.

Since the early description of the mitochondrial ultrastructure by Sjostrand (1953) and Jade (1952-1953), evaluation of changes in mitochondria by various agents has been an important task in attempts to clarify the process of cell degradation and death. Through the investigations of Friedmann & Ford (1961) and Wersall & Hawkins (1962), dark inclusions have been found in inner ear sensory cells of streptomycin-treated animals. These bodies were thought to emanate from mitochondria. In several studies on inner ear damage caused by noxious agents such as ototoxic antibiotics, sound trauma etc., signs of degenerated mitochondria have been presented. To our knowledge, however, no detailed

analysis of the gradual development of such changes have been made. As important reactions related to the electron transport chain, synthesis of proteins and storage of DNA and RNA take place within elements of the mitochondria, an alteration in structure probably reflects an alteration or impairment in function. As the mitochondria seem to be susceptible to damaging external stimuli, their structural state, in some aspects, can be taken as a parameter of the general condition of the cell.

The aim of the present paper was to investigate the effect of an aminoglycoside antibiotic, gentamicin, upon mitochondrial structure in the sensory cells of a model organ, the basilar papilla from a lizard, *Calotes versicolor*.

MATERIAL AND METHODS

The lizard *Calotes versicolor* was chosen as the experimental animal. The animals were kept in a humid environment at about 27°C. Gentamicin, an antibiotic belonging to the aminoglycoside group (Schlerring GMC 3 M 675) was administered intraperitoneally in doses of 150 mg per kg bodyweight per day. A few animals in the first test group received only 100 mg gentamicin per kg bodyweight per day. The morphological changes in these latter animals were the same as in the rest of the subjects of the experiment. The intraperitoneal administration was chosen for delivering the drug. Since the lizard middle ear forms a wide-necked recess from the epipharynx, intratympanic administration is not suitable. In order to study the direct and the persistent effects of the drug, the test animals were divided into two groups. The animals of the first test group were given the drug for 7 to 21 days and

sacrificed one day after the last injection. The second test group of animals were injected for 3 or 5 consecutive days. These lizards were sacrificed 21 days after the last injection. 26 animals were included in the first group and 7 in the second. After sacrificing the animals the skull was rapidly bisected and the medial aspect of the temporal bone was removed. The membranous labyrinth was

ing to current methods (Rhodin 1954, Wersall 1956, Luft 1961, Reynolds 1963) and mounted on one hole copper grids. The sections were examined in a Siemens Elmiskop I or Jeol 100C transmission electron microscope.

RESULTS

Normal anatomy

The basilar papilla, the hearing organ of the lizard, is an oblong organ about 425 μm in length. It harbours about 225 sensory cells divided into two populations. The ventral population consists of about 50 cells. These cells are equipped with short sensory hair bundles; they are unidirectionally polarized (Bagger Sjöback and Wersall, 1973). A tectonal membrane covers this cell population. The dorsal cells have tall sensory hair bundles and are bidirectionally polarized. This population has not been shown to have a distinct covering. The sensory cells are cylindrical or flask shaped and have a length of up to about 25–45 μm (Bagger Sjöback 1976).

Mitochondria are present throughout the cytoplasm and are especially abundant close to the plasma membrane in the area below the cuticular plate and in the infranuclear part of the sensory cell. They are also often present close to the synaptic areas. No mitochondria are normally seen in the sensory hairs or cuticular plate.

Mitochondrial structure. The mitochondria show variations in shape and size within the sensory cells. Spherical or ovoid and elongated forms are common. Although most mitochondria have a diameter of about 0.5–1 μm , some can be up to 4 μm in length (Fig. 1).

Most mitochondria have the orthodox con-

figuration with the outer compartment defined and the matrix more electron than the cytoplasm. The configuration of cristae is somewhat variable even within the same cell. Some cristae extend over the diameter of the mitochondrion (Fig. 2), angulation of the cristae may appear in treated sensory cells. In the elongated mitochondria the cristae are often arranged longitudinally (Fig. 3). Matrix granules may appear singly and rarely, there are two or more mitochondrial cross sections.

Fig. 2 Normal elongated mitochondrion in an animal.

elongated mitochondria dominate parts of a section in the left lower part of the micrograph. The sensory cell is in the center.

(N)

Fig. 3 Normal elongated mitochondrion in an animal. Note how the cristae traverse the whole of the mitochondrion. An afferent nerve ending (N) and a synaptic area are also present.

Fig. 4 Early degenerative phase with swollen cristae.

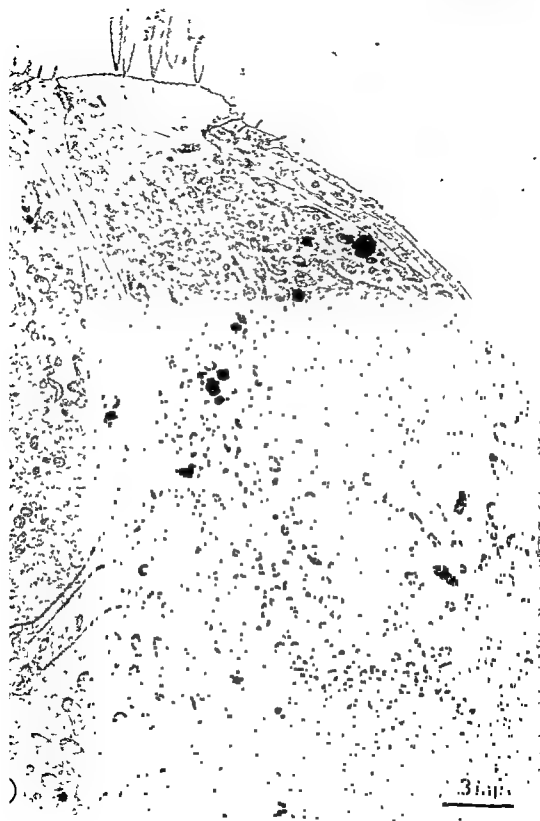
characterized by irregular karyoplasm as described in the text.

ment 8 days.

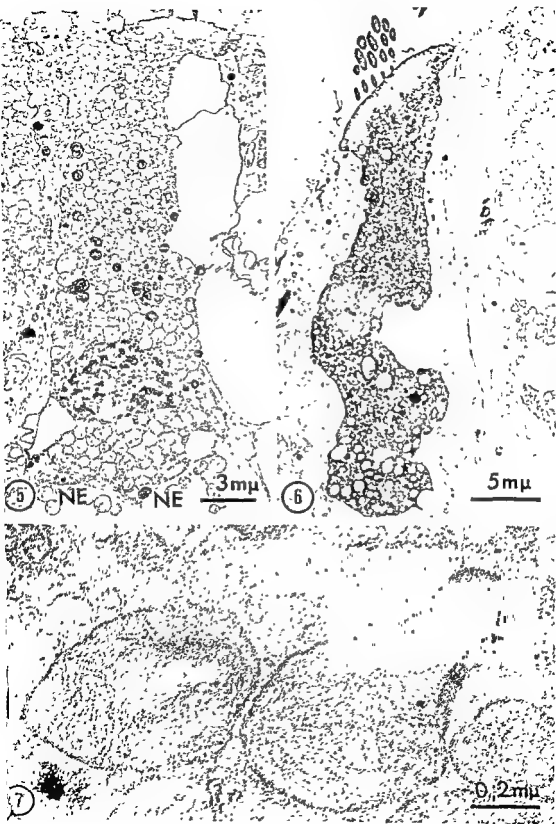
Fig. 5 Darkly staining sensory cell with irregular nucleus. The nucleus is degenerated and the cytoplasm is filled with vacuoles and mitochondria in state of degeneration. Compare the features with those of the sensory cell to the right. Gentamicin treatment 8 days.

Fig. 7 Mitochondria from the dark staining sensory cell in micrograph No. 6. The left and central parts of the cell.

These lamellae have the regularity of a normal myelin sheath; thus this phase seems to be the transition from morphologically normal mitochondria to a myelin figure. Gentamicin treatment 8 days.







Changes in mitochondria after gentamicin intoxication

Direct effects Seven days of treatment with gentamicin in some sensory cells may result in vacuolization, however the individual variation is great. Many cells appear normal while others show typical signs of degeneration. The mitochondria usually have a normal distribution. A swelling of the mitochondria in the changed cells results in a rounded or ovoid instead of an elongated appearance. Some electron-dense inclusions the size of mitochondria may also be seen in some sensory cells (Fig. 4). A marked increase of the matrix granules is evident (Figs 9, 10, 15). The mitochondrial cristae are often crescent-shaped and arranged in concentric rows (Fig. 14). These general changes became more pronounced during the consecutive days of treatment. In the more severely damaged basilar papillae, the variation between changes in individual sensory cells is striking. Large intercellular vacuoles compress and distort the outlines of the cells (Fig. 5). Most mitochondria are rounded and only rarely are elongated forms seen. Some sensory cells are almost completely filled with mitochondria in different stages of degeneration (Fig. 5). Electron dense myelin figures may be present singly or in aggregates but do not dominate the field. Most mitochondria have the orthodox configuration while some are more or less empty with only few cristae remaining (Fig. 8). Other cells have a pronounced electron density (Fig. 6) and contain mitochondria, in what appears to be a transformation phase. In other sensory cells most mitochondria appear swollen and show a loss of inner structures (Fig. 8). Few, if any, cristae remain and the matrix appears empty.

By studying mitochondria from different parts of the same sensory cell and different sensory cells in the same basilar papilla, a certain pattern of mitochondrial degeneration can be observed. Apart from a varying degree of swelling, the mitochondrial cristae in some

areas of the mitochondria lose their contours. A dense substance accumulates between the membranes of the cristae and between the cristae until a blurred area is formed in the mitochondrion. Later, parallel lines with equal intervals appear in this dark substance, so-called myelin lamellae. Thus an area of the mitochondrion can be completely altered, whereas another area can still contain normal-appearing cristae. The changes proceed until finally the whole mitochondrion

Fig. 8 Rounded swollen mitochondria with marked loss of internal structures. Gentamicin treatment 8 days.
Fig. 9 Disrupted mitochondrion with herniating cristae.

like structures are frequently seen.

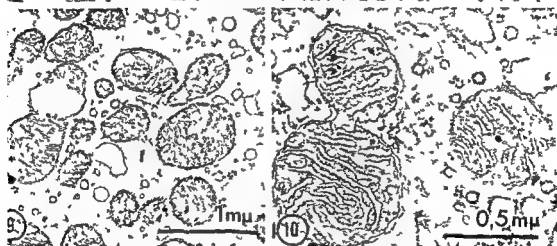
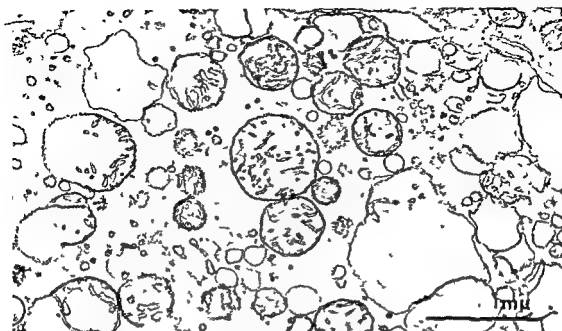
Fig. 10 Mitochondria with signs of fine interconnecting filamentous membranes. Gentamicin treatment 14 days.

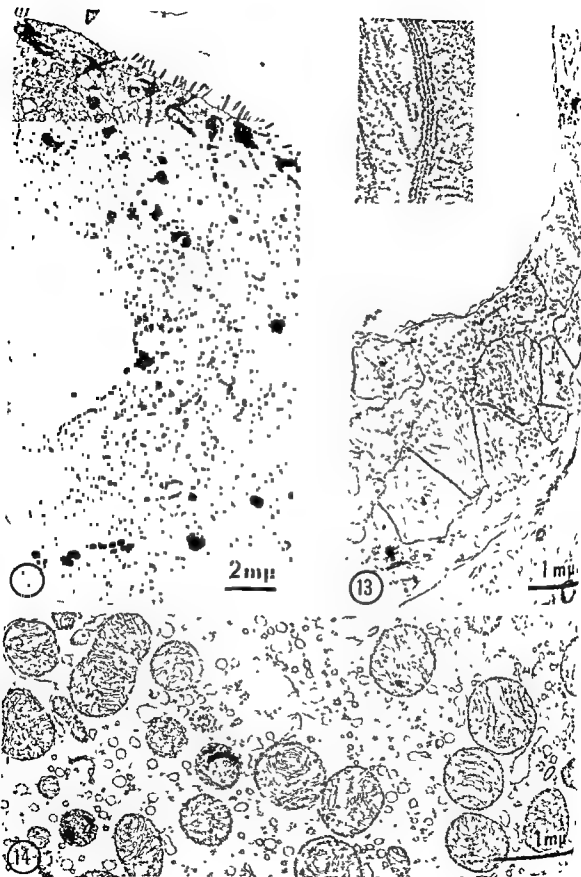
seems to separate it into two compartments. Gentamicin treatment 14 days.

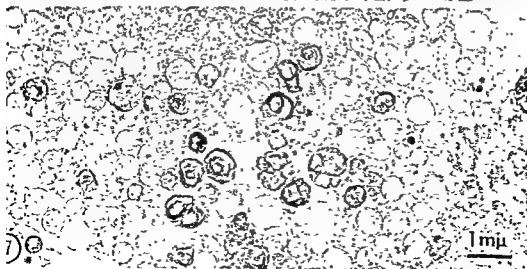
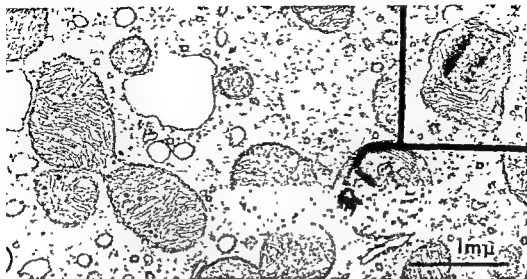
Fig. 15 Waist lined mitochondrion with 7 distinct myelin inclusions in the section. Note the angulations of cristae and the beginning lamellar arrangement of organelle in the right lower part. Gentamicin treatment 11 days. The inset shows a mitochondrion with a large inclusion that seems to dislocate the cristae. Gentamicin treatment 11 days.

Fig. 16 Large complex mitochondria with internal aggregates of rounded or spiral shaped cristae. Gentamicin treatment 11 days.

Fig. 17 Advanced cellular degeneration with very swollen mitochondria and several myelin figures. Aggregates of two or more disintegrating organelles are seen contained within a membranous bag. These bags are thought to have an autophagocytotic action. Gentamicin treatment 13 days.







changed into a dark body richly provided with myelin lamellae (Fig 7). The thickness of the lamellae in the osmium fixation is about 70 Å (Fig 7, inset). In some sensory cells "onion-like" structures with or without a central core of an amorphous material and the same size as the mitochondria make the appearance (Fig 11). These structures become more abundant as the cell changes progress. After further treatment the mitochondria may show a *partial vacuolization* where some parts appear normal and others swollen. *Matrix granules* are abundant (Figs 10, 15, 16), often seven or more granules are seen in one mitochondrial cross section (Fig 15). Most granules are round with a diameter of about 200–300 Å, but some reach a diameter of 0.5 µm and displace the cristae (Fig 15, inset). The latter type of inclusion however is rarely seen in the sensory cells. Some mitochondria are indented and may be nearly bisected (Fig 10). Often a bridging crista is seen in the narrow waist (Fig 14). *Angulated cristae* are seen in the treated as well as in the untreated ones. However the degree of angulation in the treated is greater and may exceed 90°. These angulations sometimes render a zig-zag appearance to the cristae (Figs 14, 15). Angulation of cristae is common in mitochondria of long term treated animals (Figs 10, 11, 14, 15, 16). Circular or crescent shaped cristae are rare in untreated animals but common in treated specimens, particularly in animals treated 10 days or more (Figs 11, 14, 16). Often several spirals or aggregates of cristae are present in the same mitochondrion (Fig 16). In some mitochondria the cristae may even be branched.

In animals treated for about 2 weeks, signs of more severe damage are present throughout the basilar papilla. Large intercellular vacuolization, protrusions from the cellular surface into the endolymphatic space and fusion of the hairs are common. The sensory cells are distorted and the cytoplasm contains a rich supply of small vesicles (Fig 12). Most mitochondria are swollen and in different stages of de-

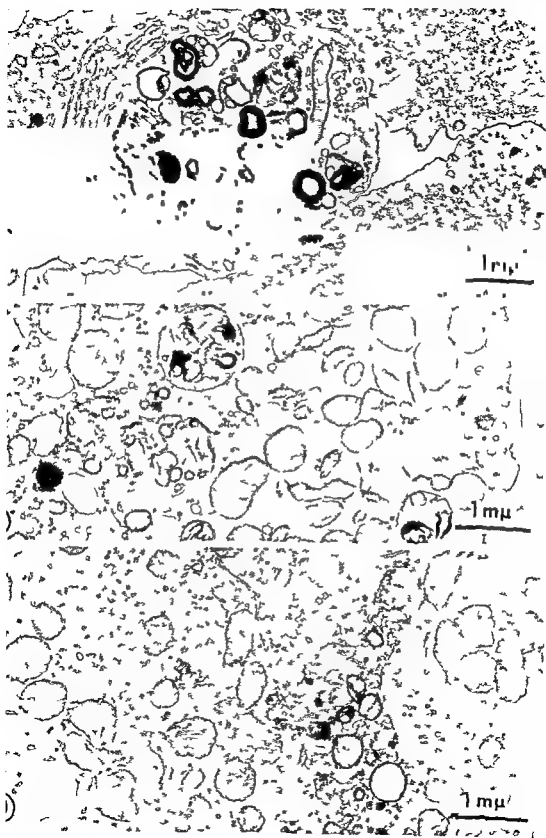
generation. The matrix has a lower electron density and elongated forms are Myelin figures and "onion like" structures common. Concentric membranes are throughout the cytoplasm (Fig 17) and contain cellular material. Aggregates of myelin figures or cellular debris are often enclosed in a membrane (Figs 17, 18). Such structures interpreted as autophagocytotic and they vary in size and shape. Often remnants of cristae are seen in them (Figs 17, 18). In some specimens, mitochondria have a tendency to establish a direct contact with one another. In the round mitochondria only a part of the periphery is involved. In other cases the mitochondria appear angulated and more square. These mitochondria are in direct contact with each other (Fig 13). The membranes of two adjacent mitochondria do not fuse but leave a gap. This gap has a constant width of about 140 Å. In the osmium fixation the gap seems to be traversed by filaments (Fig 13, inset).

After treatment for over 2 weeks the basilar papilla is severely damaged. The sensory cells may be largely absent and with progressive degeneration, the surface of the basilar papilla becomes dominated by the supporting cells. The sensory cells can be detected below the organ surface and are dominated by phagocytotic bags containing aggregated debris, membrane whorls or myelin figures and amorphous structures. Even in the

Fig 18 Severely damaged remains of a sensory cell. Membranous "bags" containing cellular debris and myelin figures dominate the field. However fairly normal looking endoplasmic reticulum is

the second test group showing persistent electron dense mitochondria with angulated or semicircular cristae evident as well as aggregates of cellular debris and myelin figures contained in "autophagocytotic bags". Gentamicin treatment 3 days with 21 days latency.

Fig 20 Short term animal of the second test group showing sensory cells in much the same state as in Fig 19. Several irregular mitochondria and a large aggregate of cellular debris is present. Gentamicin treatment 5 days with 21 days latency.



changed into a dark body richly provided with myelin lamellae (Fig 7). The thickness of the lamellae in the osmium fixation is about 70 Å (Fig 7, inset). In some sensory cells "onion like" structures with or without a central core of an amorphous material and the same size as the mitochondria make the appearance (Fig 11). These structures become more abundant as the cell changes progress. After further treatment the mitochondria may show a *partial vacuolization* where some parts appear normal and others swollen. *Matrix granules* are abundant (Figs 10, 15, 16), often seven or more granules are seen in one mitochondrial cross-section (Fig 15). Most granules are round with a diameter of about 200–300 Å, but some reach a diameter of 0.5 µm and displace the cristae (Fig 15, inset). The latter type of inclusion however is rarely seen in the sensory cells. Some mitochondria are indented and may be nearly bisected (Fig 10). Often a bridging crista is seen in the narrow waist (Fig 14). *Angulated cristae* are seen in the treated as well as in the untreated ones. However, the degree of angulation in the treated is greater and may exceed 90°. These angulations sometimes render a zig zag appearance to the cristae (Figs 14, 15). Angulation of cristae is common in mitochondria of long term treated animals (Figs 10, 11, 14, 15, 16). Circular or crescent shaped cristae are rare in untreated animals but common in treated specimens, particularly in animals treated 10 days or more (Figs 11, 14, 16). Often several spirals or aggregates of cristae are present in the same mitochondrion (Fig 16). In some mitochondria the cristae may even be branched.

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Fig 18 Severely damaged remains of a sensory cell. Membranous bags containing cellular debris and myelin figures dominate the field. However fairly normal appearing mitochondria and endoplasmic reticulum are still present. Gentamicin treatment 15 days.

Fig 19 Degenerated sensory cell in short term treated animal showing persistent effects of gentamicin. Mitochondria with angulated or semicircular cristae are evident as well as aggregates of cellular debris and myelin figures contained in autophagocytotic bags. Gentamicin treatment 3 days with 21 days latency.

Fig 20 Short term animal of the second test group showing sensory cells in much the same state as in Fig 19. Irregular mitochondria and a large aggregate of cellular debris is present. Gentamicin treatment 5 days with 5 days latency.

As the main task of the sensory cell is to induce mechanical stimulation at the hair cells to nerve impulses in the afferent system it seems reasonable that the cytoplasm near the roots of the sensory cell and the cytoplasm close to the synaptic area have certain metabolic needs. This can be said about the region adjacent to the cell membrane.

Mitochondrial swelling and early cristae changes

Mitochondrial swelling is an early sign of degeneration and may represent an unspecific change in the ion balance across the mitochondrial membranes. Persistent swelling may be due to a change in metabolic activity (Dow, 1966). Several toxic agents have been reported to induce swelling of the mitochondria as one of the first signs of damage. Inhibitors of nucleic acid replication and protein synthesis such as chloramphenicol and ethidium bromide produce swelling and loss of cristae in mitochondria of treated cells (Lenk & Pennington, 1971; Howell et al., 1971; Hwang et al., 1971). Mitochondrial swelling and loss of cristae in inner ear sensory cells was noted by Ginn & Igarashi (1969) after treatment with gentamicin sulphate. They concluded that the gentamicin had penetrated into the cells from the external surface and that the mitochondria were the initial sites of destruction. The swelling of the mitochondria in the present material is regarded upon as a rather nonspecific change. In the early stages it might be reversible. In very swollen mitochondria, a loss of the cristae may occur in the early phase of cellular degeneration after gentamicin treatment. This type of mitochondrial change probably involves an impairment of oxidative phosphorylation and possibly of respiration (Trump & Ginn,

1969). Whorls or myelin figures in inner ear sensory cells treated with aminoglycoside antibiotics were first described by Friedmann & Bird (1961) and Duvall & Wersäll (1964).

Lundquist & Wersäll found such whorls in inner ear sensory cells after treatment with kanamycin (1966) and gentamicin (1967). The whorls or myelin figures in the sensory cells of gentamicin treated animals are considered to emanate from degenerated mitochondria, as evidenced from the finding that one pole of the mitochondrion can appear morphologically normal while the opposite pole consists of a membranous whorl. Similar progressive transitions have been detected in mitochondria of the aging blow fly (Sachtor & Shimada, 1972). It was shown that the cytochrome oxidase activity was significantly decreased in the part of the mitochondria dominated by the membranous whorls. The final stage of breakdown seems to be a darkly staining multilamellar body or an "onion like" structure.

The earlier material presented by Duvall & Wersäll (1964) and Lundquist & Wersäll (1966, 1967), did not show sequences of developing changes in the mitochondria leading to myelin figure formation. In the present material we have been able to follow different steps in the degeneration of mitochondrial cristae by comparing changes in neighbouring mitochondria in the same cell as well as mitochondria in different cells in the same sensory epithelium. During this degeneration, the sharp limits of the membranes of the crista disappear. A dark substance is formed at the site of the degenerated cristae, and finally the membranes are soon to fuse forming parallel dark lines with an interval of about 70–80 Å. It seems likely that these myelin figures appear through degeneration of the phospholipid and protein complex of the cristae membranes with formation of new unit membranes arranged in a way similar to that of a myelin sheath. The multilamellar "onion like" structures have the appearance of a phospholipid vesicle as described by Ansell et al. (1973). These might thus represent the final stage in the breakdown

Myelin figures

The formation of myelin figures is under debate. Unless of their origin they are characteristic of dying and dead cells (Trump &

process of the mitochondria. An interesting observation is that these changes appear very irregularly in a cell. Thus some of the mitochondria in a cell seem to be fairly intact, whereas others are completely changed to myelin figures or 'onion like' structures. This may explain why the cells seem to survive a long time even with severe intracellular changes.

Matrix granules

Lundquist & Wersall (1967) called attention to the small matrix granules of the mitochondria in the sensory cells of the guinea pig inner ear after treatment with gentamicin. The nature of these inclusions is still not clear. Weiss (1964) and Peachey (1964) proposed that the inclusions could be sites for the binding of cations. Barnard & Lindberg (1969) and Barnard & Afzelius (1972) considered them to contain mostly lipids. The very large inclusions found in some mitochondria are probably not composed of cations but rather some kind of lipid material. Presumably the matrix granules in material is a sign of a lipoprotein unmask

in muscle mitochondria from a patient suffering from hypermetabolism. Similar changes were seen in this study and also in cells treated with chloramphenicol, ethidium bromide (Lenk & Penman 1971; Kisilev et al 1971). Tightly aggregated mitochondria as material can be correlated to mitochondrial damage. The condition has been seen in retinal cells by Pease (1962) and in cells by Pearse & Welsch (1968). A connection between the mitochondria has been suggested by Pease (1962) and believed to be by fine joining filaments.

CONCLUSION

It is important to emphasize that the action of the aminoglycoside antibiotics may be different in microorganisms and eucaryotic cells as in the inner ear of vertebrates. In microorganisms these antibiotics impair certain ribosomal functions (Lundquist 1971). Whether a ribosomal system in eucaryotic cells can be affected by aminoglycoside antibiotics is not clear. Studies on the effect of these antibiotics on cellular RNA in intoxicated cells is under way. It has been shown that aminoglycoside antibiotics are regarded to be relatively impermeable to penetrating the cell membrane (Lundquist & Sullivan, 1963) and accumulation of these antibiotics in membranes has been demonstrated in bacteria (Lundquist 1956). Hence the drugs ought to interact primarily with the surface structure of the cell, i.e. the plasma membrane. Voth & Flock (1964) demonstrated a reversal

Changes in shape and size of mitochondria

Herniations of mitochondria commonly occur in the treated cells. It is unlikely that they appear only as a consequence of the swelling process but rather that they represent an increased susceptibility of the outer mitochondrial membrane to the fixation procedure. Indented mitochondria commonly seen in many cells may be a sign of formation of new mitochondria (Larsen 1970). Further support for this idea can be derived from the heavily mitochondria laden sensory cells with moderate damage. It appears that the number of mitochondria may be increased in an attempt by the cell to cope with the decreased enzymatic capacity of the damaged mitochondria. Angulations of the mitochondrial cristae have been reported in normal cells by Revel et al (1963) and Slautterback (1965). Luft et al (1962) described angulated and spiral cristae

considered to depend on a blocking of membrane function. Wersall et al (1967) considered the membrane fusion of sensory cells to depend on an incorporation of glycophospholipids in the plasma membrane, thus increasing the surface area of the membrane. Lundquist (1976) studied the interaction of aminoglycoside antibiotics on phosphoinositide metabolism in the inner ear of the guinea pig. He suggested that streptomycin is incorporated into the mem-

its properties. The area of the plasma membrane would thereby be altered. This supports the thought that fusion of sensory hairs into giant formations may be explained by changes in membrane area. The influx and phosphorous incorporation in the membrane is connected with the phospholipid metabolism and molecular array, and this influx of calcium seems to be altered under the influence of the drugs. The aminoglycoside gentamicin is regarded by Schacht to share the action on membranes with neomycin, and to be more reactive in the cochlear tissue than in vestibular tissue. We, however, regard gentamicin to be strongly toxic to the vestibular system as well. The theory of a direct action of the antibiotic on the plasma membrane with a secondary leakage of the drug into the cell supports the findings of Bagger-Jørgensen & Wersall (1976) in their study on gentamicin toxicity in the lizard basilar papilla. The dorsal sensory cell population with long stereocilia hairs has a larger surface area than the ventral one. This dorsal cell population presented a more severe damage pattern than the ventral one. This can be explained by the rapid penetration of the drug into these cells due to their larger surface area. The steps in the degeneration of hair cell mitochondria in the present study indicate that gentamicin may have a direct action on the mitochondria of hair cells. It has been established by several authors that drugs do not introduce different structural modifications in the cell but alter the existing pathways of metabolism. Thus many toxic substances can be thought to act through a common morphologic pathway of degeneration (Forbus 1943, Trump & Ginn, 1969). Mitochondrial changes have long since been known to be a prominent feature of the degenerative process in the cells. As mitochondria are known to contain an organelle specific DNA and RNA (Nass et al. 1965) they can be assumed to enjoy a certain degree of autonomy within the cell. The inner mitochondrial membrane contains the respiratory enzymes

as well as other important enzyme systems, thus mitochondrial degeneration in the sensory cells after administration of gentamicin will severely change the functional state of the cell. In order to clarify the specific site of action of the antibiotic, histochemical and biochemical studies should be made. Further studies are also required to clarify whether or not other metabolic systems in the cell are affected by the antibiotic. Such studies are under way.

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ZUSAMMENFASSUNG

Frühere morphologische und histochemische Studien über die Wirkung von Aminoglykosid Antibiotika auf die

Anschwellung Veränderungen in der Konfiguration und Anzahl von Cristae als Bildung von Inklusionen in Mitochondrien. Myelinfiguren sind regelmässig sich wiederholende Beobachtungen in degenerierenden Zellen nach Gentamicinexposition. Deren Hauptteil stammt aus veränderten Mitochondrien. Das Endprodukt ist eine zwiebelähnliche Struktur.

Studien der verschiedenen Phasen im Abbau der Mitochondrien in den Sinneszellen konnte eine weitere Information über die Veränderungen im Zellenmetabolismus verursacht durch ototoxische Drogen erzielt werden.

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ORGAN CULTURE OF THE 16TH GESTATION DAY MOUSE LABYRINTH

A Model Suggestion for Pre- and Post partum Development

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Abstract The 16th day mouse embryo inner ear was explanted and cultured *in vitro* for 6 days which corresponds to 1 day post partum development *in vivo*. A normal development occurred both when the inner ear anlage was divided into a vestibular and a cochlear portion and when it was cultured as a whole. The present model system may provide a helpful tool in the study of both normal development and inner ear pathology.

Successful organ culture of a mammalian inner ear was first reported in 1971 by Van De Water & Ruben. Several reports have thereafter been published applying this organ culture system to the analysis of embryological mechanisms involved in normal development (Van De Water & Ruben 1973, Li et al., 1976, Van De Water 1976, 1977) and abnormal development (Van De Water & Ruben 1971). These experiments utilized eleventh, twelfth and thirteenth gestation day mouse embryo inner ear anlage and concentrated primarily upon early embryological development.

This study was designed to provide a model system for the late stages of embryological development and the post natal period of development. Preliminary organ culture results are presented in developing a late pre and post partum model system.

MATERIALS AND METHODS

CBA/CBA mice were staged for gestational ages by the vaginal plug technique with the day of plug being designated as gestation day one. Gravid females were sacrificed on the

16th day by cervical dislocation and the removed and placed in Dulbecco's phosphate buffered saline (PBS). The embryos were removed from the uterus and rapidly decapitated with a fresh razor blade knife. The heads were then dissected along the midline and the brain tissue removed. The exposed labyrinthine capsule was removed from the temporal bullae with fine watchmakers forceps. The capsule was cleaned of surrounding tissues with the watchmakers forceps and no. 25 gauge hypodermic needles that were used as microscalpels. The resultant 16th day embryo ear was either planted whole or cut in half at the level of the ductus reuniens between the saccule and the cochlear duct, leaving two explants—a vestibular explant and a cochlear explant. The explants were placed in Falcon plastic culture dishes that were coated with agar in media solution as a substrate and filled with 0.25 ml of organ culture medium. The medium was Neumann and Tytell's serumless medium supplemented with 1% L-glutamine, 1% pyruvate and 10% fetal calf serum. The temperature of incubation was 35°C and the nutrient medium was renewed every three days of culture.

Following 6 days in culture the explants were fixed with a 2% solution of osmium tetroxide.

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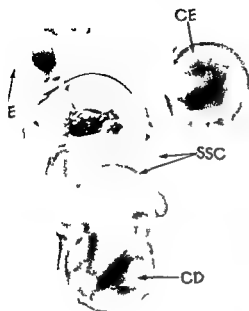


Fig 1 Light microscopy (LM) Otocyst explanted on the 16th day of gestation and cultured for 6 days in vitro Top Otocyst divided at explantation into a cochlear end and a vestibular end (VE) Bottom Whole otocyst with cochlear duct SSC semicircular canals $\times 20$

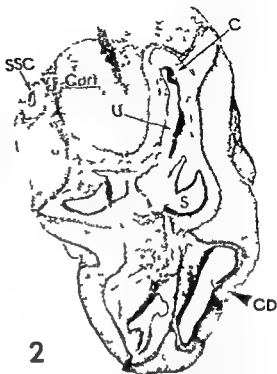


Fig 2 LM Otocyst explanted on 16th gestation day and cultured for 6 days in vitro Paraffin section through the whole explant C crista ampullaris U utricle S sacculus SSC semicircular canals CD cochlear duct Cart cartilage $\times 40$

limum phosphate buffer (pH 7.2–7.4) for 1 hour. After a rinse in buffer and dehydration, specimens were embedded in paraffin and serial sections of the whole inner ear (staining haematoxylin-eosin) were made.

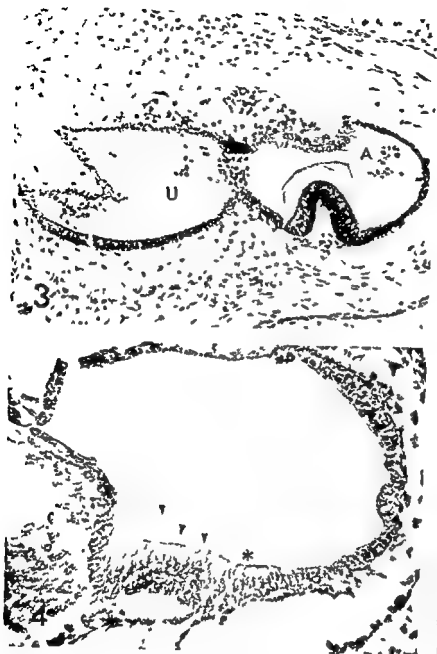
RESULTS

In culture, explants were examined daily with a Nikon dissecting microscope. The 16th day explants at the time of explantation already undergone their most dramatic morphogenesis. The only grossly observable changes were a lengthening and increased angling of the semicircular canals.

A microphotograph of living whole ear and vestibular and cochlear explants after 6 days in vitro development is seen in Fig. 1A.

A histological cross section of one of the whole ear explants is illustrated in Fig. 2. These explants showed differentiation of all of the vestibular sensory structures and base to apex pattern of differentiation of the cochlear duct. The vestibular sensory epithelium differentiated in all of the explants containing vestibular structures and sensory hair bundles and cupula formed in the explants (Fig. 3). The cochlear duct sensory areas differentiated in the explants that contained these structures. The basal turn of a cochlear duct with Corti's organ of an explant after 6 days in vitro is illustrated in Fig. 4.

This preliminary group of explants was composed of 15 specimens and a similar level of differentiation was observed in all explants as is depicted in Fig. 1 to 4.



Figs 3-4 LM Paraffin se
Otocyst explanted on 16th
day and cultured for 6 days
×90 Fig 3 A crista amp
The cupula is formed and
hair bundles are recognizable
utricle Fig 4 Basal coil of
mouse cochlea The tecton
*membrane is indicated by **
The location of cochlear ha
ir is marked by x

DISCUSSION

Ruben (1967) reported that most cells that will form sensory cells in the mouse inner ear have undergone their terminal mitotic divisions by the 16th day of gestation. The work of Sher (1971) has shown that the 16th gestation day mouse embryonic inner ear is beginning to differentiate vestibular sensory structures but has not yet begun to differentiate cochlear duct sensory structures. The developing peri-

lymphatic spaces of the 16th day embryo are encased in the cartilaginous capsule. Therefore organ culture of the 16th embryonic tissue interactions involved: differentiation may have occurred. The possibility of more prolonged organ cultures can be realized due to the presence of the developing perilymphatic spaces which may provide access of the nutrient medium to the developing sensory structures of the cochlear lymphatic lumen.

presented in the present study it is possible to maintain in short term culture the 16th day embryonic mouse inner ear and have a normal development. No adverse effects were observed as a result of the dissecting of the explants into utricular and cochlear portions. Studies are under way to establish for how long a period of time the vestibular and the cochlear parts can differentiate and then be maintained in the organ culture environment. If successful attempts at long term culture are successful, this model system might be employed widely in many experimental pathological studies, especially in hereditary diseases that previously could only be studied *in vivo*. The model may also be employed in testing the effect of ototoxic drugs on an isolated developing labyrinth.

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ZUSAMMENFASSUNG

Das Innenohr des sechzehntagigen Mausembryos wurde in vitro kultiviert, welches der 16. Tag nach der Geburt eines Tages *post partum in vivo* entspricht. Eine normale Entwicklung folgte sowohl wenn die Innenohranlage in eine vestibuläre und eine cochleäre

Partie geteilt wurde als auch wenn sie als ganzes kultiviert wurde. Das gegenwärtige Modellsystem erbietet ein gutes Hilfsmittel für das Studium sowohl der normalen Entwicklung wie der Pathologie des Innenohres.

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DIFFERENTIAL EFFECTS OF PRIMARY FIXATION WITH GLUTARALDEHYDE AND OSMIUM UPON THE MEMBRANOUS SYSTEMS OF THE STRIAL AND EXTERNAL SULCUS CELLS

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Abstract Ultrastructural preservation of the lateral cochlear wall differs between primary osmium fixation and primary glutaraldehyde fixation. Of significance in this study was the fact that whereas glutaraldehyde fixation preserves the parallel plasma membranes of interdigitations of the stria and external sulcus cells fixation with osmium occasionally produces a breakdown of selected areas of membranes into apparent vesicles. The occurrence of these membrane artifacts was more common with the external sulcus cells. Reasons for the differential effects of the fixatives may include their differing modes of action. Care should be taken when evaluating unusual ultrastructure since the type of fixative employed may be causal.

Electron microscopy is a frequently used and powerful tool for the study of the auditory apparatus. Ultrastructural detail is of critical consequence in the interpretation of both normal and pathologic cellular processes. It is not surprising, therefore, that artifacts introduced by tissue preparatory techniques are highly undesirable and can seriously interfere with the validity of experimental results.

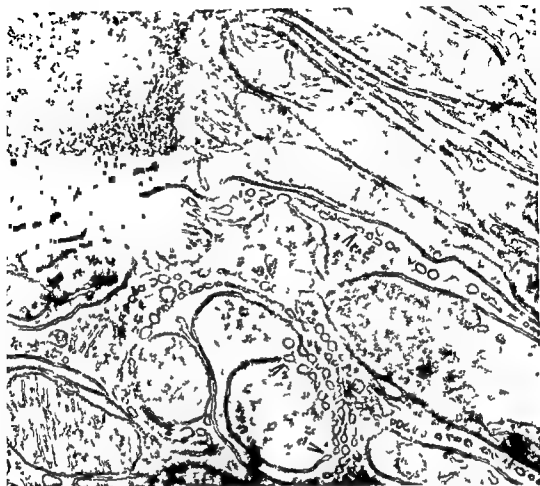
For example, most inner ear structures are quite sensitive to the amount of time elapsed from the animal's sacrifice to the application of fixative. Wersall et al (1965) have shown that if the interval is greater than 15 minutes, statistically significant ultrastructural changes occur within the organ of Corti. Such knowledge is invaluable for the proper ultrastructural interpretation of electronmicrographs.

Other less apparent factors may influence fine structure. In particular, certain fixatives are able to selectively alter cellular details. Glutaraldehyde and osmium tetroxide are per-

haps the most widely used fixatives in electronmicroscopy, either with glutaraldehyde as the primary fixative and osmium as the secondary fixative, or with osmium alone. Quite different ultrastructural anatomy may be observed with these two fixation procedures when used upon specific tissue types.

The stria vascularis is an extremely important extrasensory organelle within the cochlear duct, whose function is the production and maintenance of both the positive d.c. potential and the endocochlear unique make up (Smith et al, 1958, 1971; Spyropoulos, 1959; Bosher & Warren, 1971; Fernandez & Hinojosa, 1974). Numerous researchers have concerned themselves with the ultrastructural evaluation of the stria in normal (Smith, 1957; Hinojosa, 1966; Echarandia, 1966; Rodriguez Echarandia, 1965; Spoendlin, 1967; Sugar et al, 1971) and pathologic states (Takahashi, 1971; et al, 1974; Brummet et al, 1977).

In an evaluation of the effects of glutaraldehyde and osmium fixation upon the stria vascularis, Merck et al (1974) concluded that glutaraldehyde as the primary fixative causes various ultrastructural derangements including vacuolation of the mitochondria, cell shrinkage, and enlargement of extracellular spaces. Maunsbach (1964) reported alterations of mitochondria structure following glutaraldehyde fixation of the kidney. It was further concluded by Merck et al that the use of osmium tetroxide



Membrane artifacts (arrows) apparently budding parallel plasma membranes within the stria vascula

ris Note well fixed membranes adjoining artifacts Osmium tetroxide slightly reduced from 43 200

ry fixative produced no artifacts what
r This finding however is perplexing
the structure of the stria's cellular com
its is considered Like some other spe
ed secretory epithelia found within the
the stria's marginal intermediate and
cells exhibit numerous plasma mem
interdigitations Such interdigitations
also found within other epithelia of the
ear duct the ciliary epithelium of the
and the proximal convoluted tubules of
kidney In this context it should be noted
Formey (1964) found that fixation of the
y epithelium with osmium tetroxide as
primary fixative frequently resulted in the

fragmentation of the plasma membrane infold
ings to form rows of small clear vesicles Such
a finding was absent in glutaraldehyde fixed
ciliary epithelium Maunsbach (1966) evaluat
ed osmium and glutaraldehyde fixation in the
kidney and also found that adjacent parallel
plasma membranes of the proximal tubule
cells sometimes formed rows of small tubular
or vesicular bodies in cross section Again the
occurrence of these structures was dependent
upon a primary fixation with osmium tetrox
ide Other parallel membrane systems in dif
ferent tissues also respond in like manner to
osmium fixation These include the smooth
ER of testicular interstitial cells (Christensen

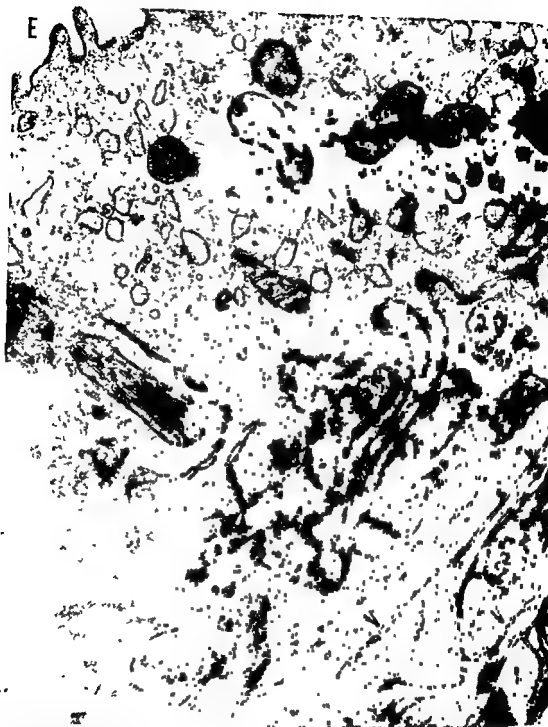


Fig 2 Membrane artifacts (arrows) close to endolymphatic surface (E) in stria vascularis. Osmium tetroxide, $\times 50300$

& Fawcett, 1961), of gastric parietal cells (Ito, 1961) and in the T system in muscle (Franzini-Armstrong & Porter, 1964)

Since the interdigitations of the strial cells are composed of parallel membranes, it is di-

stressing that Merck et al. reported differences between glutaraldehyde and osmium fixed strial membranous interdigitations. This is further evoked when one considers that various researchers have drawn art-



3 Membrane artifacts (arrows) with an external sulcus
1 Osmium tetroxide slightly reduced from 42 700

the existence of small clear vesicles occasionally found among the interdigitations of cells of the lateral cochlear wall. For example, Smith (1957) in her ultrastructural description of the stria vascularis and spiral prominence noted the occurrence of rows of small beaded vesicles apparently originating in the infolded cell membranes of the stria. Similar vesicles were found among the interdigitations of the external sulcus cells. Interestingly, the fixative employed was Dalton's

original cells contain a variable number of vesicular structures. Whether or not these vesicles were contiguous with parallel plasma membrane infoldings is not known, since no electronmicrographs of the vesicles were shown. Duvall (1969) found similar small clear vesicles in contiguity with parallel membranes of the external sulcus cells, which he interpreted as micropinocytotic vesicles. Again, the fixative used was osmium tetroxide. To this author's knowledge, vesicles of this nature within the inner ear have not been reported to occur with the use of fixatives other than osmium tetroxide. The purpose of this study then is to evaluate the effects of osmium and glutaraldehyde

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ated that the basal compartments of mar

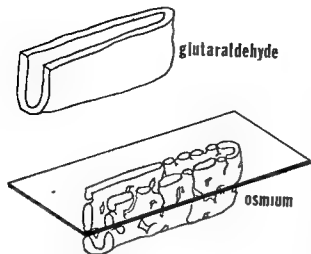


Fig 4 Schematic depiction of artifact formation and visualization with electron microscopy (redrawn from Tormey, 1964)

hyde fixation upon the parallel membranous systems of the stria vasculans and external sulcus cells, so that sound ultrastructural interpretation may be made

PROCEDURE

ng adult albino mice were used Prior to apitation, the animals were anesthetized with 25 mg/kg Nembutal Following decapitation, the cochleae were excised, fractured open and immersed either in 1% osmium tetroxide in 0.1 M cacodylate buffer or 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4°C All right ears were fixed in osmium, and left ears in glutaraldehyde The order of fixation was alternated The average interval between decapitation and fixation was 3 min for the first ear fixed and 6 min for the other After 2 hours of osmication the cochleae were washed briefly in cacodylate buffer, dehydrated in a graded series of acetone, and embedded in Epon at 60°C Prior to embedding, the lateral cochlear wall was dissected free of the bony capsule Glutaraldehyde fixation was 2 hours in length, and was followed by post-osmication for an additional 2 hours, after which the same dehydrating and embedding procedure was performed Ultrathin sections were cut with an LKB ultratome using a diamond knife

The grids were stained with saturated lead citrate, and were viewed with a Zeiss 9S-2 electron microscope

RESULTS

The overall ultrastructural appearance of primary osmium fixed specimens was similar to those fixed first in glutaraldehyde. A prominent artifact of glutaraldehyde fixation was mitochondrial vacuolation and vacuolation. Other differences between osmium and glutaraldehyde were apparent. As expected, the striae of those animals which were fixed with glutaraldehyde and osmium exhibit any breakdown of parallel membranes into rows of small clear vesicles. For the most part, membranes rather than rugosities were noted at times. Rugosities never approached discrete vesicles. On the other hand, tissue fixed with osmium alone occasionally had rows of small clear vesicles apparently budding off from the interdigitations of plasma membranes. These vesicle shaped structures were noted only in isolated areas of plasma membranes. In most interdigitations kept their parallel membranous structure after primary fixation with osmium. The isolated areas of artifact were not restricted to the deeper portions of the stria, however, since these vesicles were observed close to the endolymphatic face (Fig 2)

Whereas the appearance of artifacts derived from the plasma membranes was not noted in all stria sections examined, in some sections of the osmicated external sulcus cells contained these vesicles associated with their interdigitations (Fig 3). As in the stria, there were well fixed parallel membranes adjoining these vesicles. Glutaraldehyde-fixed external sulcus cells show no such membrane breakdown

DISCUSSION

The occurrence of small clear vesicular structures apparently budding from parallel membranes

nes of the stria and external sulcus ms to be the result of primary osmium since primary glutaraldehyde fixation d by osmium produced no such struc These observations are consistent with lts of research on other parallel plasma ine systems (Torney, 1964, Mauns 966) In some undetermined manner, i causes a breakdown of selected sec 'parallel membranes. It should be real- at whereas electronmicrographs depict down into rows of vesicles, the prob ree dimensional nature of the artifact t be vesicular, as was pointed out by y (1964) (Fig 4) Possibly during primary tion, at certain points, opposing mem coalesce to form a sheet of tubules, but when viewed in cross section, form f vesicles nsbach (1966) implied that penetration f the fixative was responsible for the ane breakdown, since he found paral-

dehyde is noted for its efficient cross linking of proteins, osmium is considered to react mainly with unsaturated fatty acids (Hayat, 1970) The inability of osmium to retain normal struc ture in the artifact zones points to the possibi- lity that membrane structure (lipidic)—and probably function too—was different at these points The lack of these artifacts with primary glutaraldehyde fixation must be accounted for by its superior protein crosslinking activity It is plausible, then, that primary osmium fixa- tion in some tissue types unmasks areas of specific membrane activity

It is interesting to note the more frequent occurrence of the artifactual parallel plasma membrane breakdown within the external sulcus cells as compared with the striae If indeed the primary osmium fixation is unmasking cer- tain functional sections of membrane, then it would appear that the external sulcus cells' in- terdigitations have a different amount of acti- vity than those of the striae

It is now clear that the vesicular structures observed by Smith (1957) in the stria and ex- ternal sulcus cells, and the "micropinocytotic vesicles" seen by Duvall (1969) in the external sulcus cells, were artifacts of primary osmium fixation Spoendlin (1967) reported the fre- quent occurrence of vesicles within the inter- digitations of the prominence stroma cells and outer sulcus cells but not within the stria The type of fixative used for the structures was not specified but presumably osmium was used for the fixation of the first two cell types, and glutaraldehyde was used for the stria The deduction that glutaraldehyde was the stria's fixative can be made because Spoendlin re- ported the appearance of small clear vesicles within the marginal cell processes These ve- sicles, about 200 Å in diameter, have been found by this author (in preparation) to be mic- rotubules in cross section, and they are only preserved with primary glutaraldehyde fixa- tion

Brummett et al (1977) studied the effects of ethacrynic acid upon stria ultrastructure, and reported that for some of their experimental

in, then penetration rates are not very nt, having K values of 0.34 and 0.2, re- vely (Hopwood, 1967a) That the rate of ation is the unlikely explanation is evi- f by the appearance of these artifacts f the stria vasculans, a tissue of about 30 width. Indeed, vesicles were observed to the endolymphatic surface (Fig 2) It f be recalled that the cochleae in this ere fractured open and immersed in e so that the fixative made direct con- nth the striae and did not have to pene- either the basilar or Reissner's mem- as in the case with the intralabyrinthine- sion technique It is difficult to account e artifacts on the basis of penetration f the fixatives used

h perhaps the explanation lies in the differing ivities of the two fixatives with membra- structural components Whereas glutaral-

animals, small clear vesicles were numerous at the basal processes of the marginal cells. Since no micrographs of the vesicles were published, it is difficult for the reader to know whether or not these were the result of the fixative used, i.e., osmium. A lack of vesicles in osmicated controls may not be significant, since not all samples may exhibit vesicles.

The differential effects of primary glutaraldehyde and primary osmium fixation on fine structure is important for the proper interpretation of electronmicrographs. This is so because effects present with one fixative but not with the other may indicate artifact formation. This is especially important for the tissues of the lateral cochlear wall, since generally poor fixation is obtained with glutaraldehyde (Merck et al., 1974) and therefore most ultrastructural work is performed with osmium as the primary fixative. Control fixation with glutaraldehyde is rarely, if ever, done. Perhaps the undesirable effects of both fixatives alone may be ameliorated by the use of a combined osmium and glutaraldehyde fixative. This procedure has been used with some success (Rump & Bulger, 1966). Until a fixation technique of superior quality is found for use on the lateral cochlear wall, care should be taken in the interpretation of unusual structures present in electronmicrographs.

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ZUSAMMENFASSUNG

Die Ultrastruktur der lateralen Cochleawand wird durch primäre Osmiumfixation oder primäre Glutaraldehydfixation unterschiedlich beeinflusst. In der vorliegenden Untersuchung konnte gezeigt werden, daß nach Glutaraldehydfixation die parallelen Plasmamembranen in der Verzahnungszone der strahlen und externen Sulcuszellen erhalten bleiben, daß aber nach Osmiumfixation bestimmte Membranregionen offensichtlich in Vesikel aufgebrochen werden. Diese Membranartefakte waren häufiger in den externen Sulcuszellen zu finden. Als Gründe für die unter-

schiedlichen Fixationsergebnisse kommen auch die verschiedenen Wirkungswesen mittel in Frage. Unübliche Ultrastrukturen sollten mit gewissen Vorbehalten interpretiert werden vom Typ des verwendeten Fixationsmittels abhängen.

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COMMENTS ON THE ACOUSTIC-REFLEX RESPONSE FOR BONE-CONDUCTED SIGNALS

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Abstract Previous studies which have measured acoustic reflex responses to bone conducted signals have not effectively differentiated reflex responses from artifacts. A convenient method for identifying such artifacts was developed and employed on some acoustic reflex measures for bone conducted signals. The findings indicated that artifacts result when a frequently used acoustic admittance meter (Grason Stadler 1720B) and a conventional bone vibrator were used to measure reflex responses for tonal and noise activating signals. It was suggested that the method be employed in future studies which investigate the acoustic reflex in response to bone conducted signals.

11 studies have suggested that the acoustic reflex responds differentially for air- and bone-conducted signals. Djupesland, Flottorp, Sundby & Szalay (1973) reported acoustic-reflex thresholds for bone conducted tonal signals which were 5 to 25 dB lower in terms of hearing level than corresponding thresholds elicited by air-conducted tones. Iwamoto & Pang-Ching (1975) reported a substantially greater percentage of acoustic-reflex responses to bone-conducted noise than to air conducted noise signals for both normal-hearing and hearing-impaired subjects. Artifacts have been reported for acoustic reflex measures for high-level air-conducted signals (Danaher & Pickett, 1974, Niswander & Ruth, 1975, Margolis & Gilman, 1977). Since acoustic energy is generated in the external auditory canals during bone conduction stimulation (Tonndorf, 1966), similar artifacts may have occurred in the above studies. Although the Djupesland et al (1973) study reported data from some control subjects which sug-

gested that their results were not inflated by such artifacts, neither of the studies effectively differentiated reflex responses from artifacts for all of the data.

The purpose of the present study was to obtain bone-conducted acoustic reflex measures employing a method which was designed to detect artifacts for each measure. A procedure was developed which conventionally measured the temporal characteristics of commonly used acoustic-impedance devices. Results obtained with this procedure were compared to the individual acoustic-reflex measures for air- and bone conducted signals taken from normal-hearing and hearing-impaired subjects, in order to separate artifacts from true responses.

Temporal Measurements of Acoustic-Impedance Devices

Several investigators have used an instantaneous change in a load impedance to measure the temporal characteristics of acoustic-impedance measuring devices. Sundby, Flottorp & Djupesland (1971) simulated an instantaneous impedance change by introducing an external probe signal into a known impedance. This method required precise control of the external signal frequency. Margolis & Gilman (1977) simulated an instantaneous impedance change by altering the current in the driver circuit of the impedance device. This method eliminated the need for an ex-

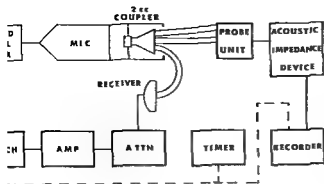


Fig 1 Block diagram of apparatus for determining temporal characteristics of acoustic impedance devices

source but still required internal access to the impedance apparatus. Morgan, Gilman & Ks (1977) produced a true change in the impedance by mechanically varying the size of a hard-walled cavity. Although the method did not require internal access to the impedance device, it was somewhat restricted in its minimum response time and did not allow for measurements of a resistive component. In addition to these specific problems, the methods required relatively sophisticated apparatus.

The method used in this paper was based on a simulated electro-acoustic change in a load impedance. It employed apparatus found in any laboratories and clinics and required no internal access to the acoustic impedance device. In addition, the method may be used to determine the temporal characteristics of simulated electro-acoustic impedance devices, regardless of the components the device measures or the probe frequency it uses.

A block diagram of the apparatus is shown in Fig 1. The probe unit of the acoustic impedance device being measured was connected to a standard 2 cc coupler (Brüel & Kjær 0138) and microphone (Brüel & Kjær 0139) normally used for routine hearing aid calibration. The microphone output was fed into a sound level meter (Brüel & Kjær 0133), an electronic switch (Grason-Stadler 7B), an amplifier (Grason-Stadler 1288), an attenuator (Grason-Stadler 1293), and a hearing aid receiver. The output of the receiver

was connected to the 2 cc coupler via the tubing on the probe unit which is normally used for varying air pressure during tympanometry. With the electronic switch turned off, the static acoustic load impedance consisted of the combination of the acoustic impedances resulting from the 2 cc cavity, the coupler microphone, the hearing aid receiver and the associated tubing. Activation of the electronic switch allowed the probe tone, generated by the acoustic-impedance device, to be amplified and directed back into the load impedance resulting in a simulated change in the static load impedance. The steady-state magnitude of the simulated impedance change was governed by the gain of the feedback circuit and was measured directly with the impedance device. Since the phase of the amplified signal was locked to the probe frequency at a constant value governed by the apparatus, the change in the magnitude of the resistive component or the reactive component was determined by the magnitude of the total impedance change. The time course of the simulated impedance change was governed by the rise and decay characteristics of the switch and a timer (Grason-Stadler 1216).

The output of the acoustic-impedance device was coupled to a complement of recording and analyzing devices consisting of an FM tape recorder (Precision Instruments PI 6200), signal averager (Nicolet 1010), and X-Y recorder (Hewlett Packard 7035B). However, any recording device, such as a storage

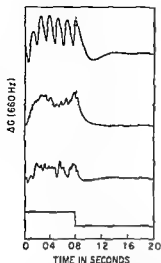


Fig 4 Change in acoustic conductance (ΔG) of the ear in response to 800 msec bone-conducted signals. Top tracing 1000 Hz. Second tracing broad band noise. Third tracing broad band noise in a subject with absent acoustic reflex by air conduction. Bottom tracing temporal course of activating signal.

sumed reflex threshold. The first tracing represents the results obtained with a 1000 Hz activator for a normal hearing subject. The

characteristics of the tracing suggest the response was due to direct activation of the measuring device. Response latencies at onset and termination and response decay times were much less than those obtained with air-conduction signals, and were similar to the temporal characteristics of the device shown in Fig 2. Two additional characteristics of the tracing suggested that the response was an artifact. First, the tracing shows a sinusoidal waveform during activation signal presentation along with a DC shift in the baseline value. The number of cycles in this waveform changed in proportion to small changes in activation signal frequency. Second, the substantial amount of overshoot at the termination of the signal was similar to the response of the measuring device. These results suggested that the response was due to direct activation of the measuring device rather than true reflex activity.

Similar results were obtained for the other normal-hearing subjects. The mean "reflex" threshold determined from these aberrant re-

sponses was 75 dB re 1 dyne for a 1000 Hz tone. Assuming that 0 dB HL for bone conduction at 1000 Hz is approximately 24 dB re 1 dyne (Dirks & Kamm, 1975), mean "reflex" threshold for the 1000 Hz bone-conducted tone was 51 dB HL. This result was identical to the findings reported by Djupeslet (1973).

The second tracing in Fig 4 shows the response of an individual subject to a bone-conducted noise signal. One-third octave band energy (centered at 400, 500, 630, 800, 1000 Hz) were attenuated in order to limit energy in the area of the 660 Hz. Again, the response latencies at the onset, termination of the signal and the decay of the response were considerably shorter than for the air-conducted signals, and were similar to those of the measuring device. The form of the activating signal also appears to be reflected in the response during presentation since the complex waveform of the response disappeared when the signal terminated. The response "decay" may have been related to a true reflex, but this cannot be accurately determined because of the presence of the artifact.

In order to optimize the relationship between the probe tone and the activating signal, additional one-third octave bands were automatically filtered from the noise-activating signal and a lower probe tone frequency was used. Even when all components below 1000 Hz were attenuated, there was still evidence of the presence of artifacts for all of the normal-hearing subjects when either the 220 or 660 Hz probe frequency were used.

The mean "reflex" threshold by bone conduction for the normal hearing subjects was 75 dB re 1 dyne for broad band noise. A comparison of these results to those for air-conducted stimuli cannot be made since the frequency response characteristic of a bone vibrator is quite different compared to that of an earphone. Nonetheless, the difference between reflex thresholds for air-conducted and noise-activating signals (Popelka & Dubno, 1975) is

■ & Wiley, 1976) was not found when bone conducted signals, again suggest that the bone conduction responses were

s
final check, reflex threshold measure were made on 3 hearing impaired subjects who represented a variety of pathologies resulted in absent acoustic reflexes both for both air-conducted tones and noise. The third tracing in Fig. 4 is a response to a bone conducted band-pass noise signal in a 58 year-old female with measurable hearing in one ear and moderate to severe sensorineural hearing loss in the other. The temporal characteristics of the tracing and the presence of a complex waveform during signal presentation again suggest that it was an artifact. Similar results were found in a subject with a unilateral conductive hearing loss and a subject with a unilateral sensorineural hearing loss. In all three groups of subjects a reflex like pattern was observed in response to relatively low intensity bone conducted signals.

DISCUSSION

Our findings suggest that the use of bone conducted tonal and noise activating signals in the acoustic reflex results in artifacts. Using the Grason Stadler acoustic admittance meter and a conventional bone vibrator, comparison of the temporal characteristics of the response to those of the device indicate that the responses were due to direct stimulation of the measurement device rather than to reflex responses.

One reason that artifacts occurred in the present study is not known. One explanation is that the measuring device detected acoustic energy generated within the ear during bone conducted stimulation. The filter response (Margolis & Gilman, 1977) of the device used in this study may be broad enough to allow direct measurement of acoustic energy generated by the activating signal.

Although the previous studies used different equipment, this explanation may also account for their findings. For example, the data of Djupesland et al. (1973) showed that differences in reflex thresholds between air- and bone conducted signals tended to increase as the signal frequency approached the probe frequency. This may be a reflection of the filter response of the measuring device. Iwamoto & Pang Ching (1975) stated that "acoustic reflexes to bone conducted noise tended to be less well-defined than to air conducted noise" and that "bone-conducted noise stimulation resulted in slight balance meter oscillations regardless of stimulus intensity" which suggests that the waveform of the activating signal was measured directly by the apparatus.

These data are not intended to imply that bone conducted stimuli will not elicit an acoustic reflex. Because of the possibility of artifacts, however, future studies should take precautions in identifying artifacts during each measure, in addition to using appropriate control subjects. Such precautions apply to clinical measures as well. Methods which rely on visual observation of meter needle deflections, such as those used in the previous studies, are inadequate for identifying artifacts since the temporal differences between artifacts and reflex responses are quite small. The method presented in this paper was intended to provide a procedure for accurate identification of artifacts. An alternative method would be to employ non acoustic measures of middle ear muscle activity such as electromyography (Zakrisson, Borg & Blom, 1974).

ZUSAMMENFASSUNG

Vorhergehende Untersuchungen über durch knochen geleitete Signale hervorgerufene akustische Reflexe.

und auf einige knochen geleitete Mittelohrmuskelreflexmessungen angewendet. Die Ergebnisse zeigen daß Scheinreflexe vorkommen wenn man einen allgemeinen erhaltbaren akustischen Admittanzmeter (Grason Stadler

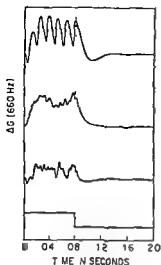


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FINE MORPHOLOGY OF THE ADVANCING FRONT OF CHOLESTEATOMA—EXPERIMENTAL AND HUMAN

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act The fine morphology of the advancing front of cholesteatoma and the mucocutaneous junction of everted skin in the feline bulla have been investigated. Inflammatory cell infiltration was frequently observed at the advancing front and the mucocutaneous junction. It is suggested that this frequent inflammation at this junction is due to the lack of a tight seal. In the advancing front, epidermal migrating cells appear to be lower than basal cells (or suprabasal cells) rather than basal cells. It appears that the slender cell processes of the migrating cells make the initial attachment to the fibrin or basement membrane left behind by the detached epithelial cells as a result of inflammation. Cells migrate along these structures, using them as a guide. Everted skin in the cat's bulla failed to develop a pearl layer but frequently developed an epiboly in which the epidermis receded and was partly replaced by the mucous membrane and its stroma was heavily invaded by mucous epithelium resembling tubular glands.

In spite of intense histopathological investigation of middle ear cholesteatoma in humans (Schuchman 1974) and in experimental animals (Riedmann 1955, Ruedi, 1959, Fernandez & Lindsay 1960) the pathogenesis remains controversial. The proposed theories are 1) congenital cholesteatoma formation from embryonic rests of squamous epithelium (Derdikman & Clemis 1974), 2) squamous metaplasia of mucosal cells to become keratinizing epithelium (Sade & Weismann 1977) and 3) migration of canal wall or tympanic membrane epidermis by marginal perforation (Bramson et al 1977) from attic retraction (Jackingham 1968) or even by infiltration of epidermal projections through the intact membrane (Ruedi 1959, Fernandez & Lindsay 1960).

Regardless of its origin, the keratinizing epithelium has to grow, migrate and expand in order to form a cholesteatoma. Therefore the modes by which migration of the cholesteatoma (epidermis) occur in the middle ear become an important research question. It is critical to determine the factor(s) which maintain the balance between the epidermis and mucous membrane or which stimulate the epidermis to migrate. The purpose of this report is to attempt to clarify some of these questions through fine morphological investigation of the advancing front of cholesteatoma and mucocutaneous junction of experimental animals.

MATERIALS AND METHODS

Seven mongrel cats were prepared for study by implantation of full thickness canal skin into their middle ear spaces bilaterally via openings in the bullae. A bed was prepared for each graft by scoring the mucosal surface. The animals were then sacrificed at varying intervals. Two each were killed at one month, three months and six months postoperatively. The temporal bones were then serially sectioned and prepared for histopathological study. The seventh cat was sacrificed at one month postoperatively and perfused intracardially with cacodylate buffered 3% glutaraldehyde and the area of skin and mucous membrane juxtaposition was dissected out and stored in the fixative overnight. The specimens were then post fixed with 1% collidine buffered with 1%

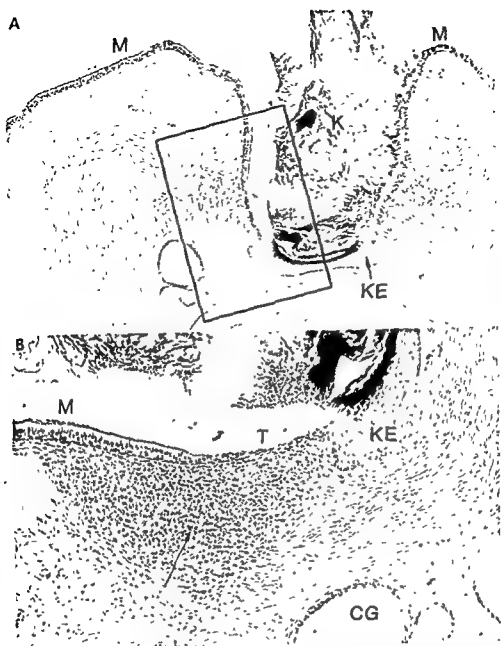
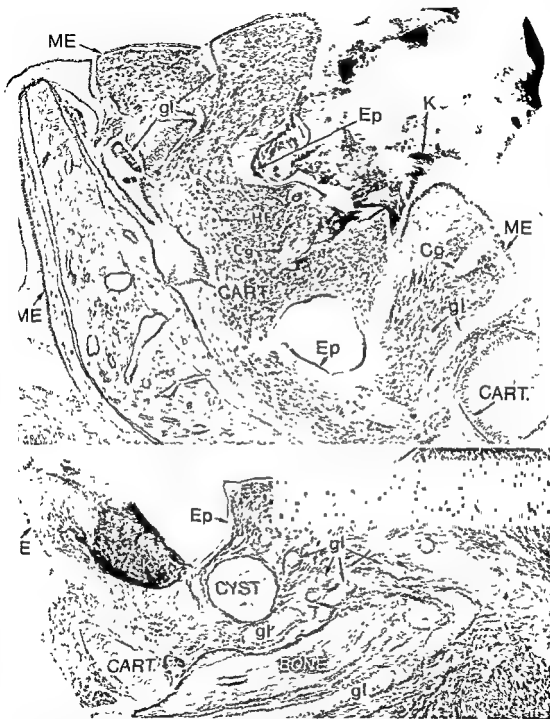


Fig 1 (A) A low power view shows mucocutaneous junction (M) C cell infiltration at the mucocutaneous junctions. An area indicated by the rectangle is magnified in (B) H & E.

×65. (B) Higher magnification of mucocutaneous from (A) shows heavy round cell infiltration between the mucous epithelium (M) and the keratinized epithelium (KE).

osmic acid for one hour and further processed for ultrastructural study, as described in an earlier report (Lim & Saunders, 1972). Fresh specimens were obtained from 5 human subjects undergoing mastoid surgery. Patients were selected for study who had cholesteatoma matrix which demonstrated an

advancing front making a confrontation the mucous membrane. Specimens obtained which would allow examination junction between these two epithelia and were processed for ultrastructure as described above with the exception fusion.



2 (A) Implanted canal skin (Ep) in the cat bulla is taken over by the mucous epithelium (ME), which infiltrated deep into the implanted skin, forming 'is (gl). Remnants of skin appendages, such as a hair (HF) and ceruminous glands (Cg), are recognizable. Cartilage (CART) of the external ear canal is recognizable 8 months after the implantation. K: keratin

H & E, $\times 30$ (B) A close-up view of the same specimen as (A) (different section) shows cystic and glandular formation (gl) of mucous epithelium beneath the epidermis (Ep) from the original implant. A portion of epidermis is replaced by mucous epithelium (ME). H & E, $\times 50$.



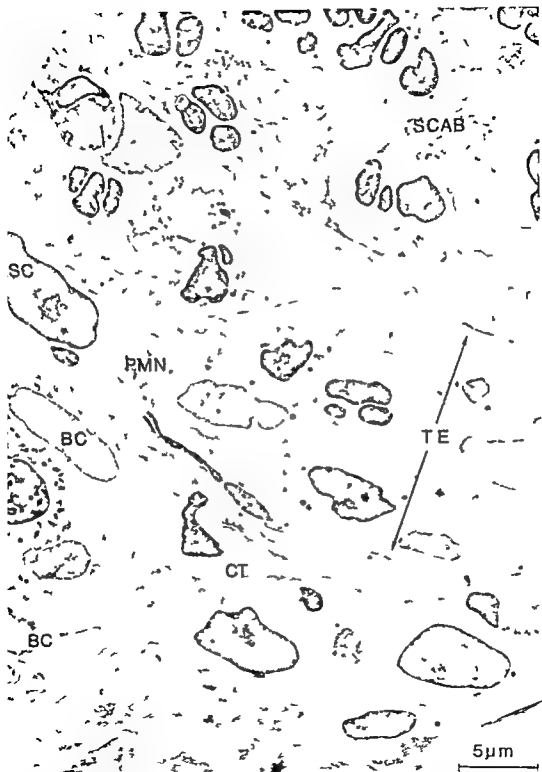
Fig 3 The inset is a light micrograph of mucocutaneous junction from a cat 22 days following skin implant in the bulla. An epidermal (KE) spur formation is bordered by transitional epithelium (TE) which is covered by a scab (S) $\times 25$. The area of the rectangle is shown in an electron micrograph which clearly demonstrates the interface between the epidermis and the transitional epithelium and scab. The epidermal spur is formed by granular (G) spinous (S) and basal (B) layers. The area of the rectangle is shown in Fig 4.

In addition, three serial-sectioned human temporal bones with cholesteatoma that were fixed with Heidenhain-Susa solution and embedded in celloidin were included in the study.

RESULTS

Animal materials

Four animals whose temporal bones were serially sectioned for study demonstrated the viability of the implants, and in the re-



An electron micrograph shows the advancing front of the epidermal spur. A lower epidermal cell (LSC) rather than a basal cell (BS) is making direct contact with the transitional epithelium (TE). Numerous polymorphonuclear leukocytes (PMN) are infiltrating the transitional cell area. CT = connective tissue.

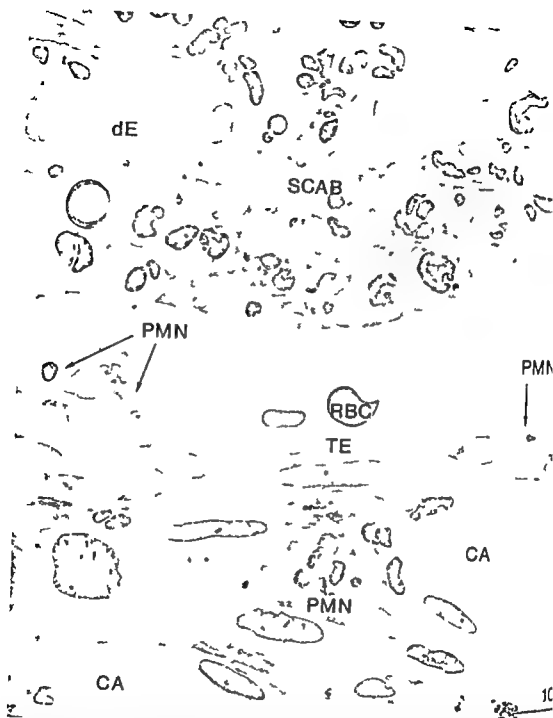


Fig 5 An electron micrograph of transitional epithelium (TE) bordering the epidermal spur shows non keratinizing pseudostratified squamous type epithelium. The transitional epithelium evidently is covered by scab which is formed mainly of polymorphonuclear leukocytes and degenerated epithelial cells (dE). A crystal in the scab is interpreted as a cholesterol crystal. CA capillary.

Fig 6 (A) A close up view of a basal transitional cell containing tonofilaments (TF), ribosomes, rough endoplasmic reticulum, and a large oval nucleus. This cell also possesses a few desmosomes (d) and hemidesmosomes (arrows). CT connective tissue. (B) A close up

view of Fig 4 where a PMN made direct contact with what appear to be migrating epithelial cells (EP) which developed hemidesmosomes with their basement membrane. N nucleus. CT connective tissue.



2 cats, which were excluded from this study, the epidermis was sloughed off. None of the animals had tympanic membrane perforation at the time of sacrifice. The implants consisted of typical stratified keratinizing squamous epithelium, made up of stratum basale, stratum spinosum, stratum granulosum and stratum corneum. The implanted skin failed to develop a typical pearl, instead it formed an epiboly. The great thickness of the connective tissue of the new mucous epithelium that encircled a patch of skin gave an impression of a cup, the bottom portion being the epidermis (Fig 1). A large portion of the epidermis of the implanted skin that formed the epiboly was replaced by a mucous membrane. The epidermis may be receding rather than invading the mucosal membrane. This interpretation is based on the fact that a thick dermis containing some epidermal appendages (such as sebaceous and cerumen glands and hair follicles) can be identified under the mucosal epithelium adjoining the epidermis (Fig 1). In addition, there was marked infiltration of mucosal epithelium resembling glandular structures inside the implanted skin, further suggesting active migration of mucosal epithelium (Fig 2).

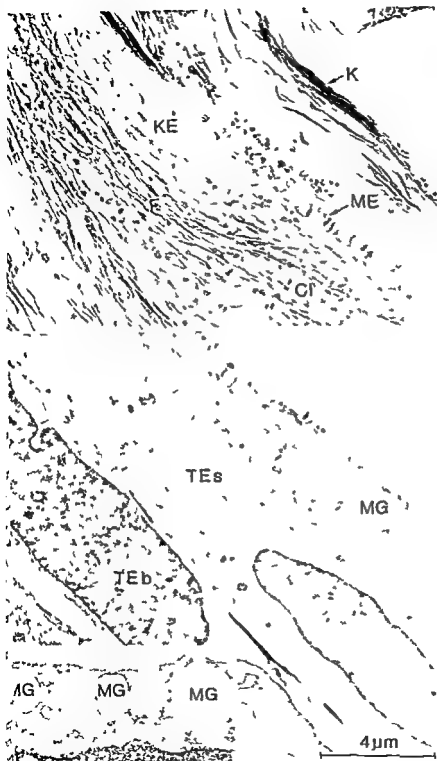
As expected, there was more evidence of acute inflammatory reaction in the animals that were sacrificed a few weeks after implantation than in those sacrificed later. In the cat which was sacrificed 22 days after the implantation a scab consisting of necrotic debris and inflammatory cells was found overlying as well as inside the mucous membrane adjacent to the mucocutaneous junction (Figs 3, 4 and 5).

The epidermis at the junction was generally thickened by hyperplasia of the stratum malpighii when there was evidence of inflammation. The hyperplasia resulted from increased cellularity, predominantly in the stratum spinosum (Fig 3). The edge of the epidermis (advancing front) resembles a wedge or plow share, which appears to be identical with the "epithelial spur" in the healing of epidermal wounds (Croft & Tarr 1970). The most distal edge of the epithelium appears to undermine

the mucous membrane (Fig 3). The fact that the stratum malpighii had advanced further into the junction than the granulosum and stratum corneum exposed surface of the epidermal edge is by flattened spinosal cells which possess numerous large cell processes. It appears lower spinosal cells or suprabasal cells (Martinez 1972), rather than basal cells migrated to the raw surface of the connective tissue adjoining the mucosal epithelium (Figs 3 and 4). The migrating cells possess scanty cytoplasm, owing to the heavy accumulation of the tonofilaments, ribosomes and glycogen particles. Although these cells do not have hemidesmosomes where they make contact with the basement membrane (Fig 6A), cell desmosomes are only infrequently seen. The migrating epithelial cells appear to have made contact with the fibrin clots by which the cells are oriented and migrate. It appears that the migrating cells may be old basement membrane that was left behind by degenerated epithelial cells.

The epithelial cells in the transition zone between the epidermal spur and the mucous membrane are also characterized by numerous processes, indicative of migration. The lamina propria underlying this area is infiltrated by inflammatory cells. Polymorphonuclear leukocytes (PMN) are abundant mostly in the lamina propria immediately beneath the junction in cases where the implant was made within a month. In one case a PMN appears to have made contact with a migrating epithelial cell giving an impression that PMN may be involved in epithelial migration (Fig 6B). Lymphocyte and plasma cell infiltration became predominant in the lamina propria with a long history of implantation. There is no remarkable round cell infiltration at the junction; the epidermal edge is thickened but still appears to underlie the mucous epithelium (Fig 7A).

The transitional epithelium closer to the mucosal epithelium became pseudostratified cuboidal epithelium or remained as



(A) Mucocutaneous junction between keratinizing epithelium (KE) and mucosal epithelium (ME) of a cat skin implanted into the bulla. The epidermis tends to undermine the mucous epithelium or the mucous epithelium migrated over the epidermis. Note that a few round cells have infiltrated (CI). A keratin

fine collagen fibers $\times 400$ (B) A superficially located transitional epithelial cell (TEs) contains secretory granules (MG) whereas the basally located transitional epithelial cell (TEsB) contains tonofilaments. An inset shows membrane-bound secretory granules (MG).

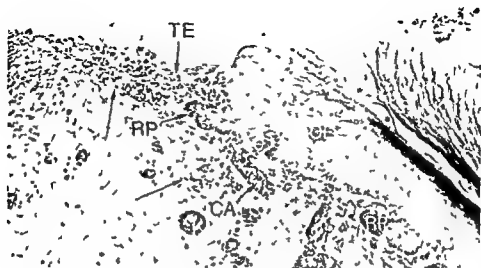


Fig 8 A human temporal bone of a patient with cholesteatoma shows that the advancing front of cholesteatoma (CE) forms an epidermal spur facing the transi-

tional epithelium (TE). Observe engorged blood vessel (CA) and exaggerated rete peg (RP) and histiocyte infiltration at the junction. A keratin

type. These transitional epithelial cells possess characteristics of metaplastic epithelial cells, containing tonofilaments in the basal cells (Fig 7B) and secretory granules in the superficial cells (Fig 7B). Even if these cells contain only a few secretory granules, their Golgi apparatus are well developed, indicating the potential of secretory activities by these cells (Fig 9). The transitional cells gradually became typical mucosal epithelium

cells thick) (Fig 9A). Some spinosa are found near the basal cells showing cell division (Fig 9B), indicating proliferation at this site. The exposed advancing front is covered by squamous type spinosal cells characterized by dark cytoplasm and cell processes on the free surface. The faces of the spinosal cells facing the cells are well covered by desmosomes. On occasion several secretory granules

Human materials

The basic morphological findings are essentially identical with those of the animals (Fig 8) except that there are no skin appendages such as were seen in experimental implants. The invagination of the rete peg is more pronounced in human specimens (Fig 8). The epidermal advancing fronts are also thickened by hyperplasia of the malpighian layer. The spinous layer contains about twice as many cells (12 cells thick) as the adjacent area (5

Fig 9 (A) A phase contrast micrograph specimen from a patient with cholesteatoma shows the advancing front facing the tissue (GT) covered with transitional epithelium (TE) and mucous epithelium (ME) $\times 250$. (B) An electron micrograph of the same specimen as (A) (3000 \times) demonstrates the face of an advancing front of keratin (A), granular cell (GC), spinosa and basal cell (BC) layers. A mitotic cell (M) in the spinosa cell layer. The adjoining connective tissue (CT) is covered by transitional epithelium (TE) which is in contact with a spinosa cell.





Fig 10. (A) Several secretory cells (SC) are attached to the spinous cells of the epidermal spur (ES). Upper rectangle is enlarged in (A) and lower rectangle is enlarged in (D). (B) Close-up view of the secretory granules (SG) from (A). (C) Close-up view of the desmosome (arrow) between a spinous cell (EC) and a secretory cell (SC). (D) Desmosomal attachment (arrow) between a cell containing secretory granules (SG) and a spinous cell with dark cytoplasm (EC).

ere observed to be attached to the free of the advancing front by desmosomes. Since we were not able to find secretory cells in the vicinity of the epidermal spur, it could be accounted for as a possible source of contamination, we have tentatively concluded that these secretory cells did not come from the neighboring mucous epithelium and have not ruled out the possibility that these secretory cells could have originated from the epidermis. The basal cells also appear thickened due to the tall columnar arrangement at the advancing edge. As in the animals, lower basal (or suprabasilar) rather than basal cells are the ones that give the appearance of active migration due to their large pseudopodia and the absence of desmosomes. At the mucocutaneous junction again are heavily infiltrated by round cells and PMN, as in the animals. The most striking finding in humans, however, is heavy infiltration of plasma cells, lymphocytes, and macrophages in the submucosal connective tissue (or lamina propria) adjoining the epidermal migrating front (Fig. 11). On the mucous membrane side of the mucocutaneous junction is again characterized by cuboidal type or simple or stratified squamous type of transitional epithelium (Fig. 12A), as in the animals, until it becomes a stratified mucous epithelium with goblet cells and ciliated cells. These cells are characterized by abundant tonofilaments, glycogen granules and/or ribosomes, and numerous cell processes (Fig. 11B). Some cells contain dark basophilic inclusions (lysosomes), as described by Martinez (1972). As in the animals, migrating squamous cells in human specimens are closely related to fibrin clots and/or basement membrane left behind by degenerating epithelial cells. It appears that migrating cells first send their slender cell processes to attach to the fibrin clots or basement membrane (Fig. 12A), and then the whole cell moves along using these structures as guides or anchors (Fig. 12B). No ciliated cells were observed in this transitional epithelium.

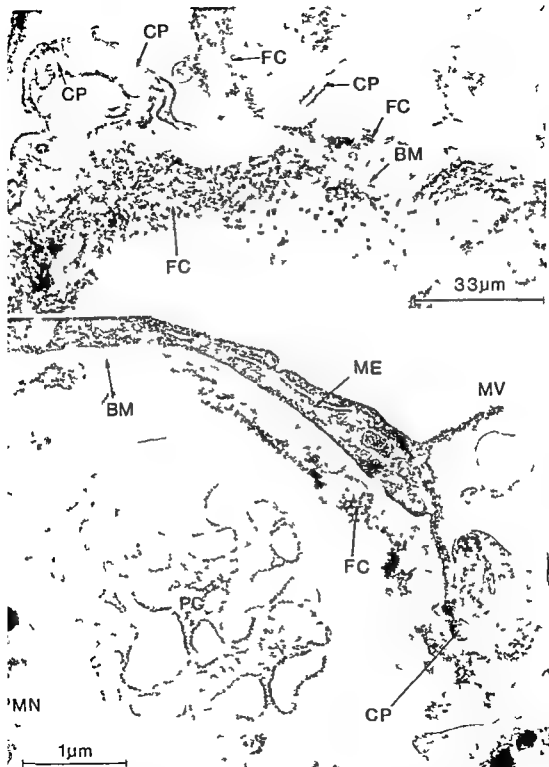
Hemidesmosomes were frequently seen in the transitional epithelial cells, but junctional complexes were absent.

DISCUSSION

The first question that we entertained in this study was regarding the behavior of the migrating epidermis and mucosal epithelium when they confront each other in the middle ear cavity. It has been established that the interaction between two epithelial cell types is governed by the phenomenon of "contact inhibition." This concept is illustrated by a classical experiment by Weiss (1959). Using phase contrast time lapse photography, he observed the random migration of epithelial cells in tissue culture. When two different cell types were combined, they tended to aggregate into colonies containing only cells of the same type. When two like cells collided, they drew closer together and remained joined. When two unlike cells met, however, they drew away from each other and continued their random locomotion. It was the ability of the cell to recognize sameness or foreignness through contact which determined its behavior. Chiakulas (1952) also made such an observation by grafting various epithelial types to wounds made on amphibian embryos. He found that when the graft was of epidermis or an epithelial type normally found adjacent to skin, e.g., oral mucosa or cornea, the migrating fronts of epidermis would meet and fuse with the edges of the graft and quickly resume a steady state. When foreign epithelium, e.g., gall bladder or lung, was grafted, however, the advancing front of squamous epithelium would migrate over or under the graft until it met and fused with the opposite skin edge. It has not been clearly established, however, whether the mucosal epithelium of the middle ear recognizes the implanted epidermal epithelium as similar or dissimilar. Since canal wall skin does not normally reside adjacent to middle ear mucosa, it can be suggested that the implanted epidermal epithe-



Fig. 11 (A) A portion of granulation tissue adjoining the advancing front of cholesteatoma is covered by transitional epithelium (TE), and the stroma is infiltrated by numerous plasma cells (P) and a few lymphocytes (L). CA: capillary; CT: connective tissue. (B) A transitional cell covering the granulation shows dark cytoplasmic dense ribosomes and abundant tonofilaments. There are very few desmosomes, but these cells possess numerous large microvilli (MV). BM: basement membrane; HD: hemidesmosomes; CT: connective tissue.



12 (A) A close up view of the advancing front of cholesteatoma where the portion of a long cell process of a migrating epithelial cell made contact with portions of basement membrane (BM) by fibrin clots

(FC) (B) A migrating epithelium (ME) has made contact with fibrin clots (FC) and oriented its direction of migration on MV microvilli CP cytoplasmic process PMN polymorphonuclear leukocytes

lum and middle ear mucosal epithelium would, upon contact with each other, perceive each other as dissimilar and remain separated.

They also may perceive each other as similar for the following reason: Experimental studies have shown that the type of underlying mesenchyme can determine the differentiation, rate of growth, and spread of embryonal epidermis (McLoughlin, 1968). It has been further demonstrated that the specificity of adult epidermal transplants is dictated by the recipient dermis (Billingham & Silvers, 1968). The fact that our implanted skin surface was partly replaced by the mucosal epithelium suggests that the connective tissue alone does not dictate the type of epithelium to be supported. Our experiment appears to support the concept that the dermis of the ear canal can also support the middle ear mucosal epithelium or vice versa. Because the keratinizing epithelium and mucosal epithelium can be derived from the same stem cells, they may recognize each other as similar cells (Peck, 1977; Sade & Weismann, 1977). The attachment of secretory cells to epidermal cells in the present study may support the idea that the secretory cells may have derived from the keratinizing epithelium, assuming that these cells had not been accidentally implanted. If this is true, then the mucosal epithelium and keratinizing epithelium should be compatible and fuse together to form a tight seal. The present findings, in general, do not support the evidence of a tight seal, as the mucocutaneous junctions are frequent sites of inflammation and/or infection. Even if they recognize each other as similar, and presumably form a seal, our study suggests that this is a very weak mucocutaneous junction. They appear to confront each other with the two epithelia competing for space in the middle ear rather than stabilizing their relationship.

It has been observed on many occasions, both clinically and experimentally, that aggressive behavior of cholesteatoma is invariably associated with infection and inflammation (Ojala & Saxén, 1968; Walsh et al.,

1951; Rüedi, 1959, 1963; Fernandez & Say, 1960; Harms, 1962; Schechter & Saunders, 1972; Abramson et al., 1977; Lim et al., 1977). Rüedi (1959) used talc as an inflammatory stimulus to the surface of the tympanic membrane of pigs, demonstrated that the inflammation seemed to stimulate basal cell proliferation and penetration of the lamina propria intact pars flaccida. Fernandez & L (1960) noted that inflammation of the dermis to ten to fifteen times normal thickness. The present investigation supports this observation in that the proliferation of the dermis is stimulated by inflammation.

Actual migration of epithelium however, independent from proliferation and has been shown in healing wounds to be inhibited by addition of mitotic inhibitors (Kroemer, 1972). The influence of inflammation, however, also seem to govern the movement of epithelium by the concept of 'contact guidance'. Weiss (1959) first described this phenomenon when he observed that connective tissue cells would elongate, align themselves with the long axis of parallel collagen fibers on the undersurface of fish scales. Cells would migrate only parallel to the fibers indicating that the migration of cells was regulated by the fibers. The leading fronts of epithelia of healing wounds also regulated by the contact guidance (Giacometti & Parakkal, 1969). It has been shown that in the initial stages of wounding an inflammatory exudate bridges the wound. The exudate contains a fibrin net which is the phenomenon of clot retraction which orient the fibers so that the major axis is parallel from wound edge to wound edge. Thus, they form a guidance system which orients migrating epithelial cells to the wound edges (Baier, 1972). It was in a histological study of epithelial cells at the wound edges parallel to fibers and seen in the

them (Martinez, 1972). We observed a cal situation at the point of confrontation between cholesteatoma and middle ear. There is an advancing front of keratinizing squamous epithelium and an inflammatory zone ahead of it. We have confirmed migrating epithelial cells align themselves the fibrin net from the transudate caused inflammation. Furthermore, we have also confirmed the earlier finding that the old basement membranes left behind by degenerated cells as a result of inflammation play an important role in new epithelial migration (Lim, 1977).

The above findings, then, are supportive of the notion that the presence of inflammation is *sine qua non* associated with cholesteatoma invasiveness, as has already been suggested (Lim & Saunders, 1972; Lim et al., 1977). Inflammation dictates this behavior, by stimulating proliferation of epidermis by forming guidance pathways directing migration. It is the inflamed stroma, or peritumour, which determines the extent of the invasive nature of a cholesteatoma rather than an inherent characteristic of the squamous epithelium of cholesteatoma itself. The reason for the frequent inflammation present at the junction in the absence of gross middle ear infection remains undetermined. It may be the effect of microorganisms which have gained access to the subepithelial connective tissue in the wound area or epithelial gap at the junction, in which case there may be a separation of the epithelia due to the lack of a tight seal at the junctional complex. However, we cannot rule out the possibility of an immunological

dermis generally receded if kept over a long period (2 months or longer) and were replaced by mucosal epithelium. Second, the transitional epithelium very closely resembles the metaplastic mucosal epithelium (non keratinizing) found in the human middle ear mucosa described earlier (Lim et al., 1977). Third, these cells contain secretory granules. On the other hand, in human specimens, where the cholesteatoma is expected to spread rather than recede, some transitional cells close to the epidermal spur may well be migrating epidermal cells. This study could not resolve the question of what factors dictate which epithelium takes over or is transformed into the other. It can be suggested that one of the factors could be the gaseous environment, such as the content of CO_2 versus O_2 , and/or humidity (Sade & Weismann, 1977). It is also possible that the basal cells of the transitional epithelium could derive from epidermal cells and the superficial cells from mucous epithelium and that the eventual survivors may be determined by the environmental factors. Further controlled experiments are required to answer this question.

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ZUSAMMENFASSUNG

Es wurden die Ultrastruktur der vorrückenden Grenzzone des Cholesteatoms und der mukokutanen Übergangszone von in die Bulla von Katzen implantierter Gehörgangshaut untersucht. Sowohl in der vorrückenden Grenzzone des

the origin of the transitional epithelial cells at the junction between the cutaneomucosal junction could not be determined with certainty in the present study. However, we suggest that these cells, at least in animals, are migrating mucosal cells rather than epidermal cells on the advancing grounds. First, the implanted epi-

Wanderzellen eher niedere Stachelzellen (oder supra

basale Zellen) denn Basalzellen zu sein. Es scheint ferner daß die schlanken Zellfortsätze der Wanderzellen den ersten Kontakt zu Fibringennetzen und/oder zu Basalmembranen herstellen, die von als Folge von Entzündung degenerierten Epithelzellen zurückgelassen wurden und daß sich die Wanderzellen dann entlang dieser Strukturen fortbewegen, sie als Leitbahnen benutzend. In die Bursa von Katzen implantierte Haut bildete keine Epithelzellen, sondern entwickelte sich häufig ähnlich wie ein Keim im Stadium der Epibolie. In diesen Fällen zog sich die Epidermis zurück und wurde teilweise durch Schleimhaut ersetzt, während in ihr Stroma schleimbildendes Epithel nach Art tubularer Drüsen eindrang.

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A COMPUTER CONTROLLED BINAURAL BALANCE TEST

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The commonly used ABLB procedure (Alternate Binaural Loudness Balance) is strongly influenced by psychological factors: the consistency of the results and the increase in intensity in equal stages on either side. To avoid this influence, side and intensity have been randomized. The test has therefore been subjected to computer control and the results are continuously displayed as to their significance. 26 patients with suspected inner ear disturbance showed a significant relative recruitment by contrast: in not one of 14 normal hearing subjects was the result positive. This test takes no longer than a conventional procedure.

There is still much controversy in the literature concerning the side of reference when performing the ABLB (Alternate Binaural Loudness Balance), (Priede & Coles, 1974, 1977). Neither of the commonly practiced procedures of this test—the method described by Jerger and that of Hood (Reker, 1972)—is in keeping with modern principles of testing, in order to avoid any psychological influence a randomization of the reference ear is required (Sachs, 1974). Such psychological effects are provoked by the fact that the intensity is fixed on the same ear throughout the entire test and that the increments are identical and thus constitute a source of error. This phenomenon can be demonstrated when testing normal hearing individuals in whom the performance of a balance test must also be possible. As mentioned above, 14 normal persons were tested, two of whom are shown in Fig. 1.

To avoid such errors, the choice of the side as well as of the intensity must be governed by random selection. The most favourable procedure is to apply pseudorandom numbers generated by a computer on line, the machine can

monitor the significance of the results throughout the entire testing procedure, and is thus in effect in control of the test.

As described by Priede & Coles (1974) the points of equal loudness are not situated exactly in a straight line but form a gentle curve. The difference between the curve and the line (Fig. 2a), which is drawn as a simplification, is so small that it cannot be proved with that number of values which one usually gets, it is essential, however, to limit the range of testing to about 25 dB above the threshold of the worse ear, as beyond this range the difference between the curve and the line widens.

By correlating all pairs of values the regression line is found, represented by the equation $y = bx + a$. 'b' is the tangent of α and thus represents the angle (Fig. 2b).

The aim of this procedure is to determine the significance of the deviation of the angle from 45° (i.e. the deviation of b from 1). To achieve this, the standard deviation of b ("s_b") is needed (Sachs, 1974). With the formula shown in Fig. 2c one is able to obtain the T-value, which represents the significance (Student distribution) of the deviation of the angle, caused by the relative recruitment.

As mentioned above, the whole procedure is computer controlled (Hewlett Packard 2100A). First the machine requires inputs. Other than data such as test number and date, both the thresholds and the maximum intensities must be stated, up to which the test can be carried out on the right, resp. the left ear. The computer then selects one of the two possible sides according to random numbers and an intensity which represents a randomized

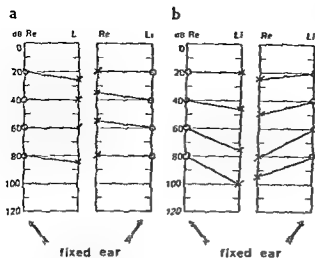


Fig 1 Balance test in normal individuals: two typical results (a) As it would be expected and often occurs (b) can also be seen frequently

value in the existing gap (this value also corresponds to a simulated Gaussian distribution). The audiometrician sets the audiometer to this value (in Fig 3a it is 54 dB for the right ear). The patient sits beside the audiometer (without being able to see the potentiometer) he himself alters the intensity presented to contralateral ear to the value (44 dB) which considers to be equivalent (a Peters audiometer AP6 was used, which can give a sweep intensity, alternate tones), in no way is the pa-

tient influenced by the audiometer value entered into the computer (the side and intensity for the next value, 3a, 56 dB to the right ear). Again the searches for the corresponding loudness for the other ear and this procedure can be repeated as often as wanted—in Fig 3a 11 times only 10–15 pairs of values have been used. The patient is searching for the corresponding loudness the computer prints out the significance, the deviation of the angle 45° and the side of the relative error. The procedure is concluded by a specific value (1E10). Finally the computer presents statistical data and draws the diagram (Fig 3b).

RESULTS AND DISCUSSION

Using the test procedure described 14 hearing persons were examined none of them showed any significant recruitment (the maximum difference was 5%). In addition both the test procedures were used on these subjects. Testing the right ear, the medium value was 0.978, on the left ear it was 1.0. The difference is significant (1% U-test). This shows that the result is dependent on side on which the tone was fixed (bec-

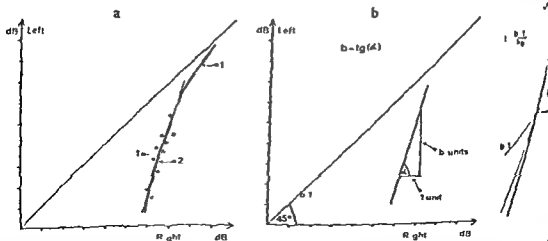


Fig 2 The principle of the test procedure (a) A line is drawn through the points which represent the pairs of values (b) This line is a negligible simplification in comparison with the curve (1) in the range 20–30 dB above

threshold (b) The value of "b" represents the standard deviation (c) The calculation of the standard deviation with help of the formula shown the calculation is possible

is a comparison of two methods both applied on the same person, this result cannot be affected by subclinical hearing disorders) It must be suspected that this psychological factor affects the result in the same way when testing persons with hearing disorders

Twenty-six patients with a unilateral sensorineural hearing loss were also tested This group of patients had been investigated with other audiological procedures, calorization and X-ray According to the anamnesis the cause of the hearing disturbance was Ménière, vertebrobasilar insufficiency (showing no retrocochlear symptoms) or unilateral noise-induced permanent threshold shift It may be assumed that in all or almost all of these cases the disturbance was located in the inner ear

The randomized test demonstrated a significant recruitment in all 26 cases and the mean value of the deviation of the angle—the difference between the regression line and the diagonal—was 14.87° ($s=5.96^\circ$) In these cases the test procedures according to Jerger and Hood had also been performed Hood's method revealed complete recruitment in 23, and Jerger's method in 8 out of the 26 cases

These results prove that the randomized test procedure with the calculation of significance is a sensitive examination procedure there was a positive relative recruitment in all patients with inner ear disturbance The test does not take any longer than the conventional procedure, its one disadvantage is the necessity of a calculator, though a machine much smaller than the one used would suffice (a portable programmable calculator such as the Hewlett-Packard 97)

Apart from using this testing method—which calculates the beginning of the curve as a straight line—it should also be possible to calculate the entire testable range as a curve This procedure may offer further diagnostic possibilities, but a considerably greater number of pairs of values would be necessary for

the calculation of a curve with accuracy

ZUSAMMENFASSUNG

Die übliche Durchführung des audiologischen

Die Zwischenergebnisse immer wieder überprüft 26 Patienten mit vermutlichem inneren hatten bei dieser Testdurchführung ein relatives Recruitment im Gegensatz dazu war von 14 Ohr gesunden das Ergebnis positiv Die verfahren dauert nicht länger als die herkömmliche führung

RÉSUMÉ

D'habitude la procédure de « ABLB » (« binaural loudness balance ») est influencée par facteurs au niveau central Premièrement l'intensité de référence ne change pas et deuxièmement l'angle d'intensité du son est effectuée en incrémentation Nous présentons une modification nouvelle de

testes Chez 26 malades avec une lésion cochléaire trouve un recrutement significatif Cependant les personnes avec des oreilles internes normales ne présentent un résultat qui était significatif pour le test Ce test modifié ne dure pas plus longtemps que la version utilisée habituellement

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SONOTUBOMETRY

An Acoustical Method for Objective Measurement of Auditory Tubal Opening

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Abstract. The sound conduction method for measurement of Eustachian tube opening has been studied and modifications made in the test procedure to increase its performance and reliability. By holding the sound source near to the nostril, mounting the microphone circumaural ear defender and by feeding the output of the microphone through a filter with a very narrow bandwidth (3.16 Hz), many of the pitfalls of the sound conduction technique described earlier could be removed. Determining of the transfer function between the nasal tip and the external auditory meatus and recording of the spectrum of swallowing sound revealed that the useful frequency range for the measurements was upwards from 100 Hz. By using the three frequencies 6.7 and 8 kHz, the method showed tubal opening in 90 or 95% of normal depending on whether the minimum amplitude needed was ≥ 5 or 1-4 dB, respectively.

In clinical practice, testing of the Eustachian tube function is performed with a wide variety of methods. Simple tests for anatomical patency alone are not adequate and should be complemented with more sophisticated methods. Much progress has been made in the development of tests for assessment of middle ear pressure and ventilatory function. However, there is still a need for simple objective methods in the evaluation of physiological tubal opening. One of the earliest established methods used for this purpose is to measure how sound is conducted through the Eustachian tube (Politzer, 1869; Perlman, 1951).

The sound source in this method was held at some distance from the subject and the sound was transmitted through a rubber tube into the nasal cavity (Perlman (1951)

pointed out that the length of the tubing connecting the ear to the microphone affected the recorded response because of resonances. This phenomenon was investigated by changing the length of the coupling tube and as a solution to some of these problems the lengths of the tube were selected so as to conform to the wavelength of the test sound used (Naunton & Galluser, 1967). The leakage of sound to the microphone directly through the connecting tube or from the sound source interfered with the evaluation of tube openings. Consequently Naunton & Galluser (1967) mounted the microphone in a 1½ inch (38 mm) thick wooden box, thus preventing any direct sound transfer into the microphone from the sound source. There were also pitfalls of possible sound leakage from the external meatus and of noise introduction into the system, if the ear plug did not give adequate seal.

Satoh et al. (1970) attached the microphone tightly to the external ear canal and the speaker hermetically to the nostril by wrapping the adapter with adhesive tape. Eguchi (1975) abandoned the use of two tubings and instead directly inserted a hearing aid earphone as a microphone to the external meatus, and another earphone as a sound source to the orifice of tubal catheter.

The frequencies of the external sound source have ranged from 60 to 2 000 Hz (Table I). Perlman (1951) stated that a tone of 100 Hz is more satisfactory for use than one of higher frequency. Tukamoto (1957) stated that any

Table I *The frequencies of delivered sound according to several authors*

Author	Year of publication	Frequency (Hz)
Perlman	1939	500
	1943	60
	1951	100
Tukamoto	1957	1 024
Elpern et al	1964	200
Naunton & Galluser	1967	200
Flach & Seidel	1967	1 000
Sato et al	1970	1 930
Cole and Cole	1974	200
Eguchi	1975	2 000
Ogawa et al	1976	2 000

frequency could be used as a test sound. He employed the test tone of 1 024 Hz because it best suited his sound source. According to Elpern et al (1964) the test frequency of 200 Hz chosen caused with their transducer less acoustic radiation outside the head than the higher frequencies and gave an additional advantage of a low attenuation in narrow tubes. Flach & Seidel (1967) used a loudspeaker as a microphone and a test tone of 1 000 Hz, on the grounds of the microphone's characteristics. On the other hand Naunton & Galluser (1967) regarded a 200 Hz sound as a good compromise between sound leakage and efficient sound conduction in their "E T Analyzer". The features of the apparatus devised by Sato et al (1970) also decided the frequency of delivered sound.

As a rule the microphones used were sufficiently good to detect the test sound at the external canal and generally the studies were carried out with the use of a calibrated condenser microphone. The microphone was connected to an amplifier and the output of an amplifier was earlier recorded directly with an oscillograph (Perlman, 1939, 1951, Tukamoto, 1957), and later also with a pen recorder (Elpern et al, 1964; Naunton & Galluser, 1967; Sato et al, 1970; Eguchi, 1975).

Although all the testing was done in a sound proofed room there was a possibility that the microphone might pick up some ambient

noise. Elpern et al (1964) first filtered the output of an amplifier (Bruel & Kjaer Type 177) with a filter set (Bruel & Kjaer Type 177) using a third octave filter with a centre frequency of 200 Hz. The -3 dB band of this type of $\frac{1}{3}$ octave filter, also used by Flach & Seidel (1967), at a centre frequency of 200 Hz is approximately 46 Hz according to IEC Publ. 225-1966 (Bruel & Kjaer catalogue 1974). The "E T Analyzer" by Naunton & Galluser (1967) and the measuring frequency 200 Hz only had a high pass filter to filter out the transients and disturbing frequencies outside of the band. It had a flat response between 100 and 205 Hz, its high and low pass rolloff was 35 dB/octave. The filter of the apparatus designed by Sato et al (1970) rejected frequencies below 1500 Hz and above 2500 Hz to prevent disturbances. The filters hitherto have had a relatively wide bandwidth so that disturbing noise could influence the response.

Because of inaccuracies in the measuring technique itself (the ear moulds were placed exactly in the same position at the time), the resonance and antiresonance effects due to the variations in the size of the ear and oral cavity, nasopharynx and pharynx because of effects of nasal abnormalities such as deviation of the septum etc. the large fluctuations of the "normal results" caused difficulties in interpreting them (Table 1). Considering these data, which are far from complete and considering the acceptance of this type of method into clinical use the present study was carried out in order to investigate the sound conduction test.

EQUIPMENT

The equipment consisted of an insert earphone as a sound source, a microphone connected into the circumaural ear defender, a frequency analyser functioning as the signal source, an amplifier and as the filter and a graphic recorder (Fig. 1). The frequency analyser

II The recorded tubal openings on normal people in different materials

	Number of Eustachian tubes	Pos itive response (%)	No positive response	Sometimes positive response
to	40	37 (80)	8	~
■ & Galluser	15	8 (53)	2	5
Se del	161	109 (68)	52	~
l tal	31	23 (74)	8*	~
Cole	28	25 (89)	1	2

*normal findings

rodyne Analyzer, Bruel & Kjaer type consists of an analyser section and a beatncy oscillator section. The output signal the oscillator is fed into the insert ear (Hearing Aid Earphone Oticon A/S AFM8) connected to a changeable olive tip held by the patient snugly in f his nostrils. It was necessary in order to a satisfactory signal to noise ratio for ound pressure level of the earphone to from 100 to 112 dB (re 20 μ Pa) as meas with the sound level meter (Bruel & Kjaer 2209) in combination with an octave set (Bruel & Kjaer Type 1613) using a ■ coupler (IEC Recommendation R 126-). The possible pressure changes in the cavity do not influence the sensitivity of earphone during swallowing and its frency response curve was nearly straight in ange of 100 to 2000 Hz with deviations rger than 3 dB (Virtanen 1977). e test sound reaching the external audi meatus through the Eustachian tube was d up by the calibrated condenser microe (Bruel & Kjaer type 4134) connected toreamplifier (Bruel & Kjaer type 2619) was embedded into the commercially able circumaural ear defender (Exel OY ta Super) (Fig. 2). The tested shielding ■ of the ear defender before mounting on an average 40 dB for frequencies in the of 2 to 8 kHz (Physikalisches Technische lenanstalt Germany 1971). In the wall of ear defender ■ thick plastic window and e the ear defender ■ small light were nt by means of which the tip of the

microphone probe could be carefully inserted into the external auditory meatus and in the other ear defender there was a hole for instructions to the patient when necessary during the examination.

The pressure frequency response curve of the condenser microphone was an almost straight line according to its calibration chart in the range of 100–10000 Hz with less than ± 1 dB error, also as controlled after completion of this study. The influence of the preamplifier response was negligible when measurements using a preamplifier were performed in the range of over 20 Hz (Bruel & Kjaer 1972). The dynamic range of the microphone and preamplifier used for the bandwidth of 3.16 Hz at 1 kHz was from 1 dB to 160 dB sound pressure level (Bruel & Kjaer 1975a).

The microphone was coupled into the external auditory meatus with a probe of suitable size. A soft standard ear tip (Madsen Electronics A/S) at the end of the microphone probe was chosen to conform to the contours of the cartilaginous portion of the individual

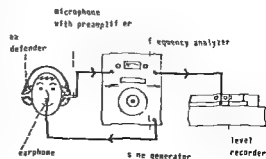


Fig. 1 A block diagram of the equipment used in the present study. See text.

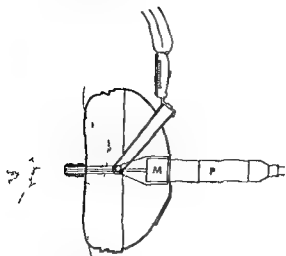


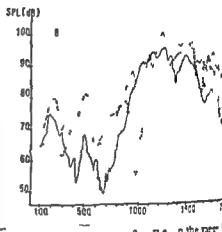
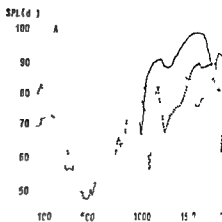
Fig. 2. A sketch of the ear defender with a microphone (M) and a preamplifier (P).

external auditory canal and was slightly compressible, ensuring a comfortable seal. However, obtaining a truly air tight seal comparable to tympanometry was not necessary.

The amplified output of the microphone was fed through a 3.16 Hz bandpass filter of the frequency analyser in order to suppress background noise and the sound pressure level in the filter section was recorded by the level recorder (Bruel & Kjaer type 2307). By using various combinations of writing and paper speeds, writing speed values of 1250 mm/s and paper speed values of 10 mm/s were chosen, permitting a sufficiently clear record of the essential features of the response. On the other hand, the filter function concerned with narrow band analysis results in a small amplitude error and time delay in writing out peaks (≈ 1 dB) (Bruel & Kjaer 1975b). By means of the event marker facility of the level recorder, operated manually by remote control, the swallowing moments could be recorded.

PRELIMINARY EXPERIMENTS

In order to evaluate the mechanics of sound transmission through the Eustachian tube during swallowing and to interpret the acoustic phenomena related to tubal function as meas-



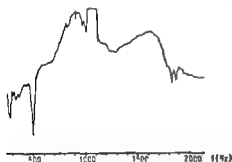
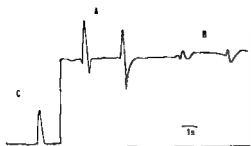
continuous line represent the same subject

ured from the ear canal, the following measurements were carried out:

1. Transfer function between nostril and ear canal in the range of 100–2000 Hz

A sinusoidal test sound was delivered to the nostril through the nasal olive and the frequency response curve was recorded in the range of 100–2000 Hz from both ears on 13 subjects and also from the other ear in 11 subjects by Heterodyne Analysis (Bruel & Kjaer type 2010) without swallowing. The total normal adult group being 18 subjects. The subjects were sitting still in a chair without moving their heads or opening their mouths. If the nasal olive was in both ears, the mouth was slightly open for testing.

The frequency response curves were



The tubal opening response as expressed with the tone of 400 Hz (A), 500 Hz (B) and without the test tone (C) and the frequency response curve in the range of 400-2000 Hz as measured from the ear canal of the same person

form in both groups but the levels of the curves were different depending upon whether the test sound was picked up from the ipsilateral nostril or from the external auditory meatus. Fig. 3 shows the frequency response curve between 100 and 2000 Hz for the nostril (A) as recorded from the ear canal and (B) from the other nostril. In the same way a woman with a constantly patent Eustachian tube on one side was studied and the response curves were basically alike in the ear canals and the nostril. There were resonance and antiresonance peaks in all the recorded curves and they varied a great deal even in the same subject in successive measurements. The form of the transfer function was influenced primarily by the nasal and oral cavities, the nasopharynx and the Eustachian tube, not so much by the middle ear cavity or the external auditory meatus as shown by Eguchi (1975) too.

Fig. 4 shows how the test sound of 500 Hz (B) will be attenuated while the one of 400 Hz (A) describes very well the time of the tubal opening in successive swallowings. The tubal opening response without the test tone (C) describes a component of 400 Hz from the swallowing sound, that was able to pass through the filter. On the frequency response curve of the same person a proportionate antiresonance peak can be seen exactly at the point of 500 Hz.

2 Spectrum of swallowing sound

The frequency analysis of the swallowing sound itself was measured from the external auditory meatus in 14 normal adults, 7 men and 7 women. All had a negative history of recent airway disease and normal tympanic membranes. Each person was asked to swallow either a sip of water or saliva several times while sitting in a relaxed position, quietly and with the mouth closed. The sound of swallowing was picked up by the microphone inserted into the circumaural defender and a third octave spectrum was analysed by means of Real Time 1/3 Octave Analyzer (Bruel & Kjaer type 3347) from both ears of each subject. This analyser performs a continuous real time analysis. The integration time of the analyser was set so that the complete event was averaged and captured on the display.

The principal shapes of the curves in all the swallowings were alike as seen on the display screen. Of these recordings a random sample of 35 spectra was chosen and recorded with the level recorder. The average was produced from the bargraphs and Fig. 5 shows this mean curve. According to this experiment the swallowing gave rise to broad spectrum noise. The variations in the spectra of different swallowings of the same subject could be due to the volume changes of head cavities during the act of swallowing. The variability was greatest in the range of 100-2000 Hz while above 7 kHz there were no frequencies on which the sound pressure exceeded the level of 30 dB.

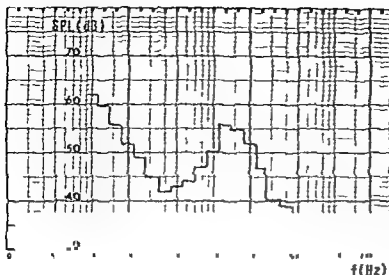


Fig 5 An average third-octave swallowing sound recorded by 1/3 Octave Analyzer from the external ear canal

The frequency response curves measured from the external ear canal and nostril were similar in form to the recordings of swallowing sound in the range 100–2000 Hz. Thus, the swallowing sound is also influenced by the resonance and antiresonance effects of nasal and oral cavities, hypopharynx and pharynx. Based upon these data the frequencies most favorable for the Eustachian tube opening measurements would be on the high frequencies, above 5–6 kHz, where the intensity of swallowing sound is weakest.

To clarify the usefulness of the high frequency test tone in connection with the mere swallowing sound, the latter with tones 0.5

and 8 kHz were recorded on the same subject by using the Real-Time 1/3 Octave Analyzer. Fig 6 shows that the test tone of 0.5 kHz cannot be seen while the test tone of 8 kHz appeared separately from the current swallowing sound. Broadband noise was used as the test sound. The resonance and antiresonance characteristics of the head alter the frequency spectrum of the sound so that it is in form similar to the current swallowing sound alone and together with broadband noise (Virtanen 1977). It is obtained supporting evidence that the frequency curve depends on the resonance effects of the head, the

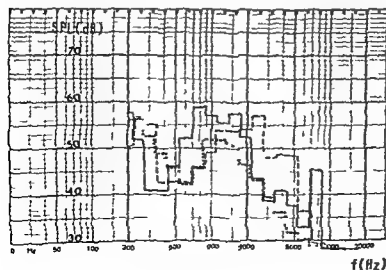


Fig 6 Sound spectrum as recorded by Real Time 1/3 Octave Analyzer from the external ear canal of the same subject. The dotted curve results from the current swallowing sound only, the interrupted curve from the swallowing sound with a test tone of 500 Hz and the solid curve from the swallowing sound with a test tone of 8 kHz.



Tubal opening responses in 42 otologically normal persons in frequencies 1–20 kHz

sis of noise was recorded on a subject who was able to keep the tube open voluntarily and on a subject who had a patulous tube. All the curves registered were alike in shape as compared with the situation of the closed tube, although there would be a direct open communication for the noise through the tube between the nasopharynx and the middle ear (Virtanen, 1977).

Tubal opening response in frequencies 6–20 kHz

Based on the above-mentioned result using the same frequencies for the sound conduction method, measurements of the tubal function were made in the range 1–20 kHz on 42 normal persons with an age range between 6 and 55 years (mean age 32 years). The intratympanic pressure of each ear was determined with tympanometry resulting in normal V shaped tympanograms in all cases. Since both Eustachian tubes were tested in each individual the total number tested was 84. After delivery of the test tone to the nose the subjects were asked to swallow a sip of water and then saliva. This was done at least once at all frequencies between 6 kHz in the range 1–20 kHz. In the analysis of the results a positive opening response was registered if there was an increase of 5 dB or more in the sound pressure level at the moment of swallowing. It is probable that by using this criterion some smaller responses are overlooked, but open excursions are easily distinguished from the background

activity recorded from the external auditory canal.

Fig. 7 shows the results of this material and it can be seen that the tubal opening was recorded best when using the frequencies 6, 7 and 8 kHz.

Eleven Eustachian tubes of 8 individuals (13%) showed no distinct opening response measured at 6, 7 and 8 kHz. In five of these 11 Eustachian tubes there was a level increase of 2 to 4 dB and the opening response was easily distinguished from the flat base line of the background. In the remaining six Eustachian tubes (7%) it was not possible to record any kind of opening response in frequencies of 6, 7 and 8 kHz, but two out of these six had a positive opening response in higher frequencies. Thus only four (4.8%) had no measurable opening response in the range 6–20 kHz during swallowing at the moment of examination.

4 Test-retest reliability

Thirty six subjects (72 normal functioning Eustachian tubes) in the normal group were

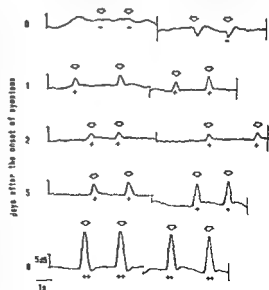


Fig. 8 Opening of the Eustachian tube from the same ear during the course of nasopharyngitis. Swallowing (of water and saliva alternately) is denoted by an arrow and subjective hearing by a plus sign. The base line is 30 dB SPL at 7000 Hz.

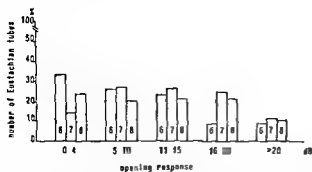


Fig 9 Opening responses of 106 normally functioning Eustachian tubes (on 60 subjects) classified according to the differing increases in level in frequencies of 6, 7 and 8 kHz

studied in detail to test the reproducibility of the sonotubometric configuration. The time between two examinations varied from 3 days to 11 months and the test tone was 7 kHz. The difference of 0-5 dB in sound pressure level for tubal opening response between the two examinations was seen in 79% and the difference seen most frequently was 0-1 dB. The difference was greater than or equal to 11 dB between two examinations in 2.8% only. These results can be interpreted to show that within subjects the test-retest consistency is quite good.

Sensitivity of test

In order to verify the accuracy of this principle, experiments were performed during the course of nasopharyngitis. Twelve persons with upper respiratory infection without earache, their ages ranging from 8 to 52 years, showed subjectively and objectively distinct signs of catarrhal condition in the middle ear: the tympanic membrane was injected, retracted, or there was fluid in the middle ear. Six of these patients had no hearing loss in their audiogram, an 11 year old boy had the largest conductive hearing loss, i.e. 10-20-10 dB in the speech area. Three subjects had normal tympanograms, 4 had a flat curve and 5 had V-shaped tympanograms the peak extending to 80 mmH₂O of negative pressure. None of these subjects (12) could open their Eustachian tubes tested by the tone of 7 kHz during swallowing. Fig 8 shows the opening of the Eustachian tube during the course of

nasopharyngitis in one of the subjects. By little, following the resolution of infection, the opening of the Eustachian tube turned to normal. An observation was made that no opening response of the tube could be recorded if an otherwise normal subject reported a respiratory infection the following day. It is thus possible that even in a subclinical stage of an upper respiratory infection the tube does not function in the normal way.

Next the tubal opening of the subjects who had the negative intratympanic pressure was recorded. This material consisted of 21 and 24 Eustachian tubes, the age range 7-27 years, 9 males and 12 females. In 14 cases with the intratympanic pressure from 10 to 15 mmH₂O no opening of the tubes was recorded. These observations are in agreement with those of Holmquist (1973). His results indicated that middle ear pressure levels outside a normal range of ± 25 mmH₂O show poor Eustachian tube function.

These experiments performed during upper respiratory tract infection test the sensitivity of this method and show the importance of investigating only patients without catarrhal symptoms in order to obtain true tubal opening results and to avoid negatives. The same conclusion has been reached previously by Flisberg (1963a, b), Andreasson & Ivarsson (1971) and Williams (1975).

Sonotubometric Results using 6-8 kHz Tones in Normal Ears

This material consisted of 106 ears of 60 subjects. The subjects ranged in age from 7 to 52 years, with a mean age of 32 years (22 males and 38 females).

After the patient had received the instructions the test tones of 6, 7 and 8 kHz were in turn applied to the nostril for 10 seconds and the patients swallowed 20 ml of water from the mouth when the tube was switched on a red light so that the swallowing moment was recorded on paper.

marker of level recorder. After each swallow of water the patient swallowed saliva when instructed by the examiner. This judgement was repeated three times at each frequency, 6, 7 and 8 kHz. The subject experienced a characteristic increase in loudness of the test tone during tubal opening. After three water and three saliva swallows the microphone probe was changed to the opposite external auditory canal and, by entirely using the same nostril, the procedure was repeated. The maximum amplitude of these six swallowings was selected for analysis of results from each side. Level changes larger than or equal to 5 dB over the baseline were accepted, because the smaller changes could not always be distinguished with certainty from the fluctuating base line.

In tubal opening recordings measured in frequencies of 6, 7 and 8 kHz (from the normal Eustachian tubes on 60 individuals) were subdivided into five groups according to the increase in level (Fig. 9). No response (≥ 5 dB) could be registered in 35 (58%) for 6 kHz test tone, in 14 (23%) for 7 kHz test tone and in 25 (42%) examined ears for 8 kHz test tone, and there was no demonstrable response (≥ 5 dB) in 11 (18%) ears for frequencies of 6, 7 and 8 kHz test tone. In 6 (10%) out of these 11 revealed tubal opening for the steady sound of higher frequencies only. In the remaining 4 Eustachian tubes (33%) no opening response could be registered (≥ 5 dB) at all during swallowing. The peak height of 1–4 dB was accepted as tubal opening provided it was easily distinguished from the base line signal; no opening response occurred in only 5% of normal ears during swallowing tested with 6, 7 and 8 kHz.

COMMENT

In earlier reports on the sound conduction method have presented it as valid as used in routine testing (Sato et al., 1970). Its problems including a larger number of false responses (11–47%) have hampered its

acceptance. The other serious disadvantages of the sound conduction technique described earlier were its susceptibility to the different background noises, the arbitrary choosing of test frequencies and the fluctuation of the base line signal. Many pitfalls existed, like the leakage of the sound through the tubes, the badly fitting ear plug lacking ability to achieve an airtight sealing in the external auditory meatus and the touching of the microphone tube. The testing demanded a sound proofed booth and the microphone had to be shielded against noise.

By holding the sound source near to the nostril, mounting the microphone into circumaural ear defender and by feeding the output of the microphone through a filter with an extremely narrow bandwidth (3–16 Hz) many of these pitfalls could be removed. There was no need for a sound proofed booth, even the instructions could be given to the patient during the examination when necessary, because the microphone system so constructed attenuated the background noise by more than 30 dB and because the level of high frequency components in the normal speech spectrum is low.

The recording of the transfer function of the passage between the nasal tip and the external auditory meatus revealed that some frequencies were attenuated while the others were amplified. This unequal attenuation of various frequencies could depend on specific physiological changes in volume and shape of the nasal cavity, the oral cavity, the nasopharynx and the pharynx. The recording of the spectrum of swallowing sound speaks in support of the existence of this resonance and antiresonance phenomenon. This explains (Fig. 4) why in earlier investigations there were many negative or sometimes debatable responses in otherwise otologically normal individuals.

It became obvious from the present experiments that the useful frequency range for the measurements by the sound conduction method was narrowed from 5–6 kHz. This was

presented in Fig 6, where the delivered tone of 500 Hz unsatisfactorily detected the tubal opening response, while the test tone of 8 kHz was easily distinguishable from the base-line. One may reasonably expect that various kinds of resonance effects of head cavities also influence the higher frequencies (>5–6 kHz), but the wider dynamic range here makes it possible to record the acoustic phenomenon. It is obvious that one simple frequency may not always give a reliable picture of the tubal opening, if the result proves negative. By using the frequencies 6, 7 and 8 kHz (Fig 7) positive results (≥ 5 dB) showing a tubal passage were obtained in about 90% of normal ears. If the peak height of under 5 dB is accepted for tubal opening, provided it is easily distinguished from the base line signal, the opening response occurred in about 95% of normal ears during swallowing. In the normal group there can also be persons whose middle ear aeration is not achieved by swallowing but for instance by moving the mandible, by yawning, by blowing nose and by the Valsalva manoeuvre. In Elner et al (1971) had cases (6.9%) in material of 102 ears which seemed to use a technique other than swallowing for equilibrating.

Perlman (1939) showed by means of sound conduction method that the tube does not open regularly to the same extent during each act of swallowing. His recordings are in accord with the present study, but as a rule the tubal opening responses seemed in this material to be rather similar in the same patient during the separate swallowings, although each subject had a swallowing sound pattern of his own. It may be that some persons can swallow better with a sip of water than with saliva but the pen excursions were of similar magnitude for both water and saliva swallowing. Similar conclusions have also been made by Cole & Cole (1974) and by Williams (1975).

ZUSAMMENFASSUNG

Die Methode der Tondurchleitung zum Messen der Öffnung der Eustachischen Röhre wurde untersucht und das

schutzes eingebaut und der Output des Mikrophons geleitet wird konnten viele Mängel der Technik die früher beschrieben worden waren werden Tonaufnahmen der Übertragungen von den Nasenspitzen und dem äußeren Gehörtrakt Aufzeichnungen des Spektrums des Schalls gaben einen nützlichen Frequenzbereich für Swallowings von mehr als 5–6 kHz. Bei einer Reihe von drei Frequenzen 6, 7 und 8 kHz zeigte eine Tubenöffnung bei 90 oder 95% der normal je nachdem ob als minimale Amplitude ≥ 5 dB entsprechend 1–4 dB angenommen wurde.

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LONG-TERM OBSERVATION OF EARS WITH REDUCED MIDDLE EAR PRESSURE

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Abstract 335 ears from 210 children in which tympanometry initially indicated middle ear pressure ≤ -100 mm H₂O or a flat tympanogram have been reinvestigated 3 years later. When the initial investigation was performed all children were 7 years old. The present study reveals that 25% of these ears still have middle ear pressure ≤ -100 mm H₂O and 16% middle ear pressure ≤ -150 mm H₂O. In normal material of the same age group 9% have middle ear pressure ≤ -100 mm H₂O and 4% middle ear pressure ≤ -150 mm H₂O. The study also reveals effusion in 10% of ears with normal screening audiograms. In ears with normal screening audiograms and middle ear pressure ≥ -145 mm H₂O effusion was found in 5% and in ears with abnormal screening audiograms and middle ear pressure ≤ -150 mm H₂O effusion was found in 81%. As tympanometric criterion for otitis media with effusion middle ear pressure ≤ -150 mm H₂O or a flat tympanogram is suggested. The inclusion of tympanometry in routine screening for middle ear pathology in children is recommended.

Diagnosis and treatment of serous/mucoid otitis media is a routine problem for all otologists. Many authors believe that serous otitis media predisposes the ear to adhesive processes, chronic otitis media and cholesteatoma. To prevent development of such chronic processes, early identification of this disease is certainly important. Previous studies have demonstrated Eustachian tube dysfunction and subnormal middle ear pressure in all ears examined during serous/mucoid otitis media and in approximately 70% of the ears examined after this disease (see for example Renvall, 1975). However, the long term importance of reduced middle ear pressure is not yet clear.

The aim of this paper is to report a 3 year

follow up of ears in which subnormal middle ear pressure was demonstrated during serous/mucoid otitis media. This observation led to the development of middle ear pressure criteria for use in evaluating serous otitis media.

MATERIAL AND METHODS

The material of this investigation is comprised of 210 children. When the initial investigation was performed all children were 7 years old and at evaluation they were between 10 and 11 years old. The ear with a middle ear pressure of ≤ -100 mm H₂O or a flat tympanogram at the initial investigation was included in this study. Screening audiograms were obtained in all ears and were considered pathological if air conduction thresholds exceeded 20 dB in two or more ears. The middle ear pressure as well as the impedance was measured with a Madsen impedance tympanometer (probe frequency 270 Hz). The pressure in the ear canal was varied between -400 and +70 mm H₂O during the tympanogram recording. The upper limit of the hearing threshold was determined by stimulating the ear with a tone burst of 1000 Hz and a duration of 0.1 s. The stapedius reflex threshold was determined by stimulating the ear with a tone burst of 1000 Hz and a duration of 0.1 s. The hearing threshold was determined by stimulating the ear with a tone burst of 1000 Hz and a duration of 0.1 s.

stapedius reflex threshold of >95 dB HL was considered abnormal. Otolologic examination of the ear was performed by an experienced otologist.

RESULTS

Otolologic evaluation of these 335 ears revealed pathology (retracted or stapedius fixation, effusion) in 25% of the ears examined. Tympanometry and audiometry showed normal findings in 16% while the stapedius reflex threshold was pathological in 61% of the ears. See Table I.

I Findings in 335 ears in which tympanometry 3 years earlier demonstrated middle ear pressure ≤ -100 mm H₂O or a flat tympanogram

	Normal	Abnormal
Effusion	(75%) 250 ears	(25%) 85 ears
Audiogram (normal)	(84%) 282 ears	(16%) 53 ears
Tympanometry	(84%) 282 ears	(16%) 53 ears
Stapedius reflex	(39%) 129 ears	(61%) 206 ears

Effusion related to audiogram and middle ear pressure

Effusions were observed in 10% of 282 ears with normal audiograms and 51% of 53 ears with abnormal audiograms. Tables II and III show our basic observation of the relationship between effusions to otologic and audiologic findings. In those ears (256) with both normal middle ear pressure (≥ -145 mm H₂O) and normal audiograms, effusion was observed in 5% of the cases, while in those ears (27) in which a pathologic middle ear pressure (≤ -150 mm H₂O) and abnormal audiogram were identified, effusions were observed in 81% of the cases. Effusions were found in 54% of ears (26)

Table II Middle ear pressure and otologic findings in 282 ears with normal screening audiograms (a) and in 53 ears with abnormal screening audiograms (b)

Effusion revealed by otoscopy in 27 ears of those with normal audiograms (10%) and in 27 ears of those with abnormal audiograms (51%)

H ₂ O	No. of ears	Effusion (otologically)
Normal screening audiograms		
≥ -145	(91%) 256	(5%) 13
≤ -150	(9%) 26	(54%) 14
	282	27
Abnormal screening audiograms		
≥ -145	(49%) 26	(19%) 5
≤ -150	(51%) 27	(81%) 22
	53	27

Table III Tympanometric and otologic findings in 335 ears in which tympanometry 3 years earlier indicated middle ear pressure ≤ -100 mm H₂O or a flat tympanogram

mm H ₂ O	No. of ears	Effusion
≥ -145	282	6%
≤ -150 or flat tympanogram	53	68%

demonstrating pathologic middle ear pressure (≤ -150 mm H₂O) and normal audiograms and 19% of ears (26) with abnormal audiograms and normal middle ear pressure.

Effusion related to middle ear pressure

Tympanometry revealed abnormal middle ear pressure (≤ -150 mm H₂O) in 16% in the present investigation (Table I). Six per cent of these ears with normal middle ear pressure (282) and 68% of ears with abnormal middle ear pressure (53) demonstrated effusions (Table III).

Analysis of ears with subnormal middle ear pressure and of ears with effusion demonstrated at myringotomy

Initial selection of cases for following study was based upon a criterion of a middle ear pressure ≤ -100 mm H₂O. As is shown in Table IV, 25% of the ears still demonstrated a middle ear pressure of this value or less. Judged by our new criterion, 16% were pathologic.

Table IV Results from follow-up of 335 ears in which tympanometry 3 years earlier demonstrated middle ear pressure ≤ -100 or a flat tympanogram compared with the findings in a nonselected group of 10-year-olds (206 ears from Renvall et al., 1973)

Middle ear pressure (mm H ₂ O)	Selected group (no. of ears)	Nonselected group (no. of ears)
≤ -100	(25%) 84	(9%) 19
≤ -150	(16%) 53	(4%) 7

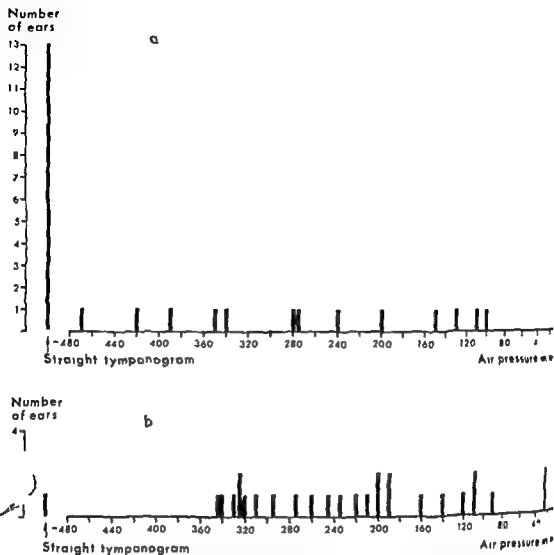


Fig. 1 (A) Tympanometrically recorded middle ear pressure in 27 ears 1-2 hours prior to myringotomy. Effusion could be demonstrated in all ears. (B) Tympanometrically

recorded middle ear pressure in 28 ears 1-2 hours after myringotomy. No effusion could be demonstrated.

logical. In 55 ears in which the otologist recommended myringotomy, tympanometric recording was performed immediately prior to surgery. As seen from Fig. 1 a straight tympanogram is more common in ears with surgically verified effusion than in ears with out effusion. However, 10 ears with effusion demonstrated a recordable negative middle ear pressure of ≤ -150 mm H₂O and in 4 ears with effusion, the middle ear pressure was > -150 mm H₂O.

In the present investigation one ear has developed cholesteatoma and 13 ears retracted

tympanic membranes or myringo-
pexis (Table V) even though treat-
ment of the ears in question was performed in
conjunction with the initial investigation.

DISCUSSION

The diagnosis and treatment of serous
otitis media has been the subject of great
discussion during the last decade (Brodie
& Djupesland, 1969; Ferrer, 1974; Földi
& Wagoner, 1974; Müller & Geier, 1974; K
et al., 1975; Lewis et al., 1976). In 1974

V Otologic findings and screening auditory in 53 ears with middle ear pressure 0 mm H₂O or a flat tympanogram

	No of ears	Normal	Retracted ear drum or myringo-stapedio-peris-	Effusion	Cholesteatoma
Admission	26	2	10	(54%) 14	
Final	27	1	3	(81%) 22	1

on culminated in a conference entitled 'Recent advances in middle ear effusion', in which early detection of this disease was considered essential (Paparella, 1975). Since pure audiometry alone is not an adequate method for diagnosis of serous/mucoid otitis media (Brooks, 1968, Renvall et al., 1973, Jerger & Wagoner, 1974, Renvall et al., 1975), interest has been directed toward the development of new diagnostic techniques. The introduction of impedance measurement procedures in a form appropriate for routine clinical application has added a valuable diagnostic tool whose full potential still remains to be established.

In this investigation it was shown that reduced middle ear pressure can be found in the presence of a normal screening audiogram. It is appropriate to question whether these children, who would be classified as normal on the basis of screening audiometric results, only should receive further otologic examination. In Berry's study (1975) 5 of 7 ears with effusion had a middle ear pressure of ≤ -150 mm H₂O (660 Hz probe tone, admittance meter) and 49 of 66 ears with effusion had a middle ear pressure of ≤ -150 mm H₂O (220 Hz probe tone). Our study reveals that in over half of the ears with normal audiograms and reduced middle ear pressure, effusion was found (see Table IIa). The detection of one additional child in 11 (9%) with normal middle ear function (Table IIa) would seem a sufficient argument for inclusion

of tympanometry as part of any routine battery of screening tests.

Additionally, in recently published long-term studies of ears with serous/mucoid otitis media, adhesions were shown in 11.2% (Gundersen & Tonning, 1976) and 3.3% (Tos & Poulsen, 1976) of the cases reported and the incidence of cholesteatomas varied between 5.6% (Gundersen & Tonning, 1976) and 1.1% (Tos & Poulsen, 1976). These data indicate that ears with subnormal middle ear pressure constitute a risk group for the development of chronic middle ear disease. Negative middle ear pressure of a certain magnitude may itself be responsible for development of retractions in the tympanic membrane (Tos & Poulsen, 1976) and when there is a retraction the ear has the potential to develop adhesions as well as cholesteatomas. Typically the symptoms are minimal before this state of chronic middle ear disease develops. Identification of this ear, predisposed to serious pathology, is difficult. One possible means for such identification is regular, routine evaluation of ears in which subnormal middle ear pressure has been demonstrated. Obviously, screening for such subnormal middle ear pressures is appropriate.

Three considerations must be raised in the use of tympanometry to screen for middle ear abnormality.

(1) In a temporal bone study (Renvall et al., 1975) it was shown that small amounts of water in the middle ear can change the impedance without flattening the tympanogram. It is thus obvious that if the aim is to recognize effusion in the middle ear we must consider ears with recordable middle ear pressure as well as ears with a flat tympanogram. Thus in 14 ears with effusion (Fig. 1) a dip was recorded on the tympanogram and the middle ear pressure could be measured. According to our criterion, 11 of these 14 ears with effusion showed abnormal middle ear pressure.

(2) The criterion employed in the classification of an ear as normal or abnormal is critical. We suggest that a criterion may be formulated

on the extent to which middle ear effusion is predicted by a given tympanographic finding. Berry et al., 1975, Brooks, 1969, Grimaldi, 1976 have discussed the relevance of tympanogram shape. Using as criterion a pressure of ≤ -150 mm H₂O we identified 23 of 27 ears with effusion (Fig. 1). We suggest that such a criterion is easy to apply and may be more appropriate than sophisticated analysis of tympanogram shape, particularly in the use of this measure in screening tests.

(3) Finally we note that reduced middle ear pressure can be a transient episode and require no treatment. To avoid referring inappropriate patients to the otologist, repeated determination of middle ear pressure is suggested (Brooks, 1975, Liden & Renvall, 1977).

As is shown earlier by Renvall et al. (1975) the reflex threshold is always higher than 95 dB HL or not elicitable in ears with middle ear pressure ≤ -100 mm H₂O. In this study the stapedius reflex threshold was pathological in

10% of the ears. Therefore, the use of the stapedius reflex test according to our criterion for identification of ears with effusion is considered too sensitive a test. If, however, failure to elicit the reflex at 110 dB HL on two consecutive occasions is considered to be an indication for otologic investigation, this test must be discussed. Brooks (1975) found that in an unselected material, around 15% belonged to this category. However, the relation between middle ear pressure and pathologic stapedius reflex test must be further evaluated before we can decide whether the stapedius reflex test may add significant dimension to tympanometry (or indeed replace it) in our efforts to detect serous/mucoid otitis media.

In such contralateral testing as we and others have used, it is often difficult to evaluate the extent to which each ear contributes to an abnormal finding. The use of ipsilateral testing procedures may alleviate this difficulty. With some concern for possible artifacts we are persuing ipsilateral testing procedures in our present and future studies.

As mentioned earlier in this study one ear

has developed cholesteatoma and 10% retracted tympanic membranes or a stapediopexis. The important observation, however, is that 25% of these ears still had a middle ear pressure of ≤ -100 mm H₂O and that 16% had pathologic middle ear pressure according to our criteria. In a selected group of the same age 9% had a retracted middle ear pressure ≤ -100 mm H₂O and 4% had pathologic middle ear pressure according to our criteria. (See Table 1). This follow-up shows that reduced middle ear pressure is more common in ears that had subnormal middle ear pressure in a nonselected group. Therefore regular determination of patients with subnormal middle ear pressure is recommended. This very simple procedure may be one way to avoid development of chronic middle ear disease which often results in life long dependent medical care.

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ZUSAMMENFASSUNG

Bei 335 untersuchten Ohren von 210 Kindern war Tympanogramm einen Druck im Mittelohr von ≤ -100 mm H₂O oder ein flaches Tympanogramm. Diese Kinder sind nach 3 Jahren wieder untersucht worden. Zu ersten Untersuchung waren die Kinder 7 Jahre alt. Bei den Kindern wurden nach 3 Jahren wieder eine Untersuchung unterzogen bei welcher es sich zeigte dass 25% der untersuchten Ohren weiterhin einen Druck im Mittelohr von ≤ -100 mm H₂O und 16% einen Druck von ≤ -150 mm H₂O hatten. Eine Untersuchung von normaler Gehör zeigte folgende Werte: 9% hatten einen Druck im Mittelohr von ≤ -100 mm H₂O und 4% ≤ -150 mm H₂O. In der Untersuchung befand bei normalen Tympanogrammen mit einem Mittelohrdruck von ≥ -100 mm H₂O zeigte bei 5% der Untersuchten eine Sekretbildung im Mittelohr. Bei Ohren mit abnormalem Tympanogramm und einem Mittelohrdruck von ≤ -100 mm H₂O zeigte sich eine Sekretbildung bei 16%. Die Studie zeigt dass bei Ohren mit normalem Tympanogramm eine Sekretbildung bei 5% der untersuchten Ohren vorkommt. Als tympanographisches Kriterium zur Überweisung an einen Otologen wurde ein Mittelohrdruck von ≤ -100 mm H₂O oder ein flaches Tympanogramm.

vorgeschlagen Die Anwendung der Tympanometrie
Routine Screening bei Mittelohr Pathologie ist empfehlenswert

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BACTERIOLOGY OF THE CHRONICALLY DISCHARGING MIDDLE EAR

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Abstract: Suitable bacteriological techniques revealed anaerobic bacteria in 38 (33%) of 114 chronically discharging middle ears. The genus *Bacteroides* was cultured from 25 ears. Aerobic bacteriology showed the predominance of staphylococci, facultative enteric Gram-negative rods, diphtheroid bacilli and *Pseudomonas* species. Anaerobic bacterial cultures were always mixed with aerobic bacteria. 12 ears were culture-negative and 9 of the 108 Gram-stained smears revealed no bacteria. No significant difference in bacteriology was noted between ears with or without local antimicrobial treatment or between profusely draining or only moist ears. The ears with postoperative recurrent infection or with clinical suspicion of cholesteatoma grew anaerobes significantly more often and were seldom sterile. Because anaerobic bacteria are frequently associated with chronic media, their characteristics with regard to susceptibility to antimicrobials and to air must be remembered in the choice of therapy.

MATERIAL AND METHOD

The study was carried out on 100 outpatients with active chronic otitis media in a total of 114 ears. 47 of them had been previously subjected to surgery. No dry ears were included in the study. Bacteriological specimens were taken under operation, microscopic examination at the same time, the appearance of middle ear discharge, and the status of the eardrum membrane were inspected. Since the operation, 24 of the ears have been subjected to radical surgery, and cholesteatoma was found in 18 of them.

Specimens of the middle ear discharge were taken with a silver ear probe before or after cleaning of the ear. The specimens were inoculated and spread on enriched blood agar and neomycin-vancomycin-chocolate agar plates. The plates were pre-reduced in a GasPak anaerobic system taken out 10 seconds before and placed back immediately after the inoculation. Aerobic specimens were taken similarly on chocolate agar plates which were put in a candle jar. Incubation at 37°C was started immediately. The jars were later transported to the bacteriological laboratories. Each specimen was Gram-stained. The anaerobic technique and the identification of anaerobes followed the lines proposed in the *Manual of Anaerobic Bacteriology* (Smith 1975).

The clinical findings, the results of

The improved anaerobic bacteriological techniques of recent years have led to a re-evaluation of the bacteriological etiology and emphasized the role of the anaerobes in many bacterial infections (Gorbach & Bartlett 1974). The foul smell of chronic ear discharge and the high frequency of anaerobic bacteria in otogenic intracranial infections (Heinemann & Braude, 1963) suggest that anaerobes are a common occurrence in chronic otitis media. Yet earlier methods have revealed only a few anaerobes (Palva et al. 1971) or none at all (Friedmann, 1957; Wright 1970; Decher & Daum 1973; Cooke & Raghuvaram 1974; Deha & Kacker 1975). We therefore decided to study the bacteriology of chronic otitis media by using appropriate aerobic and anaerobic bacteriological methods.

Table I Bacteria isolated from 114 consecutive ears

	No of isolations
<i>C. fr.</i>	
<i>C. des fragilis</i>	6
<i>C. des melanogenicus</i>	16
<i>C. des clostridioformis</i>	1
<i>C. des sp. *</i>	8
<i>C. terium necrogenes</i>	1
<i>C. terium sp. *</i>	1
<i>C. terium infantis</i>	1
<i>C. rum lentum</i>	2
<i>C. rum sp. *</i>	4
<i>C. bacterium acnes</i>	1
<i>C. bacterium sp. *</i>	4
<i>C. rum sporogenes</i>	1
<i>C. rum sp. *</i>	1
<i>C. ella alkalescens</i>	1
<i>C. ella parvula</i>	3
<i>C. ella sp. *</i>	1
<i>C. coccus magnus</i>	5
<i>C. coccus sp. *</i>	8
<i>C. streptococcus sp. *</i>	2
<i>C. *</i>	65
<i>C. anaerobic and facultative</i>	
<i>C. E. coli</i>	9
<i>C. E. aerogenes</i>	2
<i>C. E. bacter sp. *</i>	8
<i>C. E. sp. *</i>	1
<i>C. E. ter sp. *</i>	2
<i>C. E. mirabilis</i>	11
<i>C. E. morganii</i>	2
<i>C. E. rettgeri</i>	1
<i>C. E. monas aeruginosa</i>	7
<i>C. E. monas sp. *</i>	9
<i>C. E. philus sp. *</i>	1
<i>C. E. bacter sp. *</i>	1
<i>C. E.teroid bacillus*</i>	34
<i>C. E. subtilis</i>	2
<i>C. E. ia sp. *</i>	8
<i>C. E. coccus aureus</i>	32
<i>C. E. coccus epidermidis</i>	27
<i>C. E. coccus*</i>	1
<i>C. E. lytic streptococcus*</i>	2
	160

*Es not identified

and of the staining were all recorded separately. The χ^2 test was used for the statistical analyses between bacteriological and clinical classifications.

RESULTS

A total of 225 bacterial strains were cultured (Table I). 160 aerobic and 65 anaerobic (29%) of which *Bacteroides* accounted for 45%

Table II Gram staining in 108 chronic ears

Gram	No of identifications
Gram negative rod	64
Gram positive rod	42
Gram negative diplococcus	16
Gram positive coccus	55
Total	177

Table III Bacteriological finding and local antimicrobial therapy

Bacteriological finding	No of ears		
	Drops	No drops	Total
Anaerobic			
Mixed with aerobic	18	20	38
Aerobic			
Mixed	10	15	25
Pure	25	14	39
No growth	9	3	12
Total	62	52	114

(29/65) of the anaerobes, *Bacteroides melanogenicus* being cultured 16 times. The second in order of frequency was the genus *Peptococcus*, found in 13 cultures (20% of the anaerobes). The aerobic bacteriology showed the predominance of staphylococci (59), facultative enteric Gram negative rods (36),

stresses their importance in the infective process of a chronic ear.

Anaerobic bacteria grew in 38 ears (33%), always mixed with aerobic bacteria. Each ear contained 3-4 species on average, 1-7 anaerobic and 1-7 aerobic. *Bacteroides* species were cultured from 25 ears, that is, from 22% of all the ears and from 66% of the ears with an anaerobic bacteria. 12 ears (11%) were culture-negative.

Gram stained smears were obtained from 108 ears, in 98 (91%) of them bacteria were found (Table II). Seven of the 108 ears (6%) were devoid of bacteria both in the culture

Table IV Bacteriological finding and middle ear discharge

Bacteriological finding	No. of ears			
	Pure	Inter-mediate	Moist	Total
Anaerobic				
Mixed with aerobic	29	5	4	38
Aerobic				
Mixed	19	4	2	25
Pure	27	7	5	39
No growth	4	2	6	12
Total	79	18	17	114

and in the Gram stain. Parallel to the culture results, most of the stainable bacteria were seen in abundant numbers. By morphology, a total of 177 bacteria were traced in the smears. In 31 ears (29%) the culture and staining results agreed, in 54 ears (50%) the smears showed bacterial species not found in the culture and in 47 ears (44%) the culture revealed bacteria not seen in the smears. Altogether the stain revealed 69 bacteria not disclosed by the culture. 15 of the stainable gram negative diplococci were unverified in culture, and in other Gram groups roughly one third of the bacteria remained culturally unverified. Further, all the 12 cultured diplococci (4 *Veillonellae* and 8 *Neisseriae*) failed to stain, in other Gram groups the share was again roughly one-third. Of the 39 ears with

Table VI Bacteriological finding and perforation of the Tympanic membrane

Bacteriological finding	No. of ears	
	Attic/postero-superior	Other
Anaerobic		
Mixed with aerobic	11	20
Aerobic		
Mixed	4	17
Pure	4	31
No growth	0	11
Total	19	89

pure culture, 20 showed additional bacteria in the smears, making mono-infection very rare ($19/114=17\%$) in chronic otitis media.

62 of the ears had been treated with drops within the previous 12 hours (Table IV). This did not correlate significantly with bacteriological culture findings although there was a trend towards more abundant bacteriology in the untreated group. As regards appearance of the middle ear discharge, bacteriology was essentially the same in both groups, but sterile cultures concerned significantly ($P<0.001$) in cleaner ears (Table V). In the group with postoperative resection there were more anaerobic and sterile cultures than in the unoperated group (Table V). Both these findings were significant ($P<0.05$). Perforation of the

Table V Bacteriological finding and previous ear surgery

Bacteriological finding	No. of ears		
	Operated	Unoperated	Total
Anaerobic			
Mixed with aerobic	20	18	38
Aerobic			
Mixed	11	14	25
Pure	14	25	39
No growth	2	10	12
Total	47	67	114

Table VII Bacteriological finding and cholesteatoma (operatively verified)

Bacteriological finding	No. of ears	
	Cholesteatoma	No cholesteatoma
Anaerobic		
Mixed with aerobic	9	2
Aerobic		
Mixed	2	0
Pure	6	4
No growth	1	0
Total	18	6

membrane was attic and/or postero or in 19 ears. These showed significantly anaerobic bacteria ($P < 0.01$) and were often sterile ($P < 0.05$) than those with perforations (Table VI). 24 of the ears were operated since the bacteriological nation (Table VII). Anaerobic bacteria cultured from nine of the 18 cholesteatomas against two of the six ears with cholesteatoma. Although the groups were small to show statistical significance anaerobic bacteria seemed to concentrate in cholesteatomatous ears.

DISCUSSION

The present study confirms our recent finding (Pu et al. 1977) that anaerobic bacteria are an important component of the bacterial flora in chronic otitis media. Our finding of anaerobic bacteria in 33% of the ears contrasts with the earlier findings of up to 1.1% (Palva 1971), a common development in other studies of infections, well suggesting the inadequacy of the earlier methods of investigation (Gorbach & Bartlett 1974). Even the disk system with pre-reduced anaerobic media and bedside inoculations used may lose the most fastidious anaerobes (Schae 1969) and probably the real number of anaerobic bacteria in chronic otitis media is greater than we discovered.

This is also supported by our finding of stainable bacteria traced by the present culture method in 40% of the ears studied. However, the incidence of anaerobic bacteria was exactly the same as in the smaller material analysed by the group (Jokipii et al. 1977). These facts suggest the reliability of the method we used. Although the pathogenicity of most of the opportunistic anaerobes presented here is recognized (Zabransky 1970), the role of anaerobic bacteria in chronic otitis media cannot be established. However, in infections caused by the normal flora of the skin and mucous membranes, anaerobes are thought to be important pathogens (Gorbach & Bartlett 1974).

Chronic otitis media may be classified among these infections.

The anaerobes always grew in mixed cultures. Facultative bacteria are believed to act synergistically in mixed infections; they remove oxygen, produce substances that lower the redox potential of the tissues, or provide nutrients that are necessary for the proliferation of the anaerobic pathogens. This kind of bacterial synergy is probably common in anaerobic infections, particularly in superficial infections such as otitis media.

The bacteriology did not differ significantly between the groups with or without local antimicrobial therapy. Because the groups were not random samples, this only means that the ear drops used did not sterilize the ear. Furthermore, (anaerobic) bacteria were as frequently seen whether the ear drained profusely or was only moist.

The ears with postoperative recurrent infection or with clinical suspicion of cholesteatoma (attic or posterosuperior perforation of the drum) showed anaerobic bacteria significantly more often and were seldom sterile in the present material. Thus, anaerobic bacteria were especially associated with the ears having the most troublesome disease problems. Although it can be speculated that the anaerobes proliferate more readily in the ears with the most troublesome pathology, the relationship does not imply an etiologic role of the anaerobic bacteria in these processes.

The aim of conservative treatment in active chronic otitis media is to eradicate infection and to make circumstances for future operation as favourable as possible. This is achieved by mechanical cleaning of the middle ear and by local antimicrobial treatment. Systemic antimicrobials are needed only during some acute stages of chronic otitis media and during pre- and postoperative treatment of the ear. When antimicrobial therapy is being considered, the characteristic susceptibility of the anaerobes to various antimicrobial agents must be remembered. When the treatment is local, this feature of the anaerobes is especially

ly noteworthy. For instance, all the anaerobes are resistant to aminoglycosides, of which neomycin and gentamicin are commonly used ototopically. The different effects of these as well as many other antibiotics on different kinds on bacteria, and their suspected ototoxicity (Mittelman, 1972) offset the questionable benefits achieved by their ototopical use (Lancet, 1976). Consequently, in the treatment of chronic otitis media, the importance of careful and repeated mechanical cleaning of the discharging middle ear is further emphasized.

ZUSAMMENFASSUNG

Durch geeignete bakteriologische Verfahren wurden anaerobe Bakterien bei 38 (33%) von insgesamt 114 chronisch eiternden Mittelohren festgestellt. Die Gattung *Bacteroides* wurde von 25 Ohren kultiviert. Unter den aeroben Bakterien waren Staphylokokken, fakultative enterale gramnegative Stäbchen, Diphtheroide und Pseudomonaden vorherrschend. Anaerobe Bakterien wuchsen immer als Mischflora zusammen mit aeroben Bakterien. 12 Ohren waren kulturnegativ und bei Gram-Färbung wiesen 9 von 108 Proben keine Bakterien auf. In der Bakteriologie gab es keinen signifikanten Unterschied zwischen Ohren mit und ohne örtliche antimikrobielle Behandlung. Auch war kein Unterschied zwischen profus eitrigen und nur feuchten Ohren festzustellen. In Ohren mit postoperativ wiederkehrender Infektion oder mit klinisch suspektem Cholesteatoma wurden signifikant mehr anaerobe Bakterien beobachtet, und sie waren selten steril. Weil anaerobe Bakterien häufig bei chronischer Mittelohrentzündung angetroffen wurden, müssen ihre Antibiotika- und Luftsensibilität bei der Wahl der Behandlung berücksichtigt werden.

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COMPUTER ANALYSIS OF OPTOKINETIC NYSTAGMUS IN PATIENTS WITH SPONTANEOUS NYSTAGMUS OF PERIPHERAL VESTIBULAR ORIGIN

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The bias of slow phase velocity (SPV) of optokinetic nystagmus (OKN) caused by an acute labyrinthine lesion was examined in 8 patients using different optostimulus velocities. In all patients a directional preference of OKN-SPV was found corresponding to the spontaneous nystagmus. This was due to enhancement of the SPV to the side of the lesion and depression of the opposite horizontal direction. The preponderance of OKN on the average increased with the intensity of the spontaneous nystagmus and decreased along with regression of the nystagmus. These vestibularly induced differences in OKN-SPV up to 70%. A differentiation is discussed between OKN preponderances caused by labyrinthine and brain stem lesions.

Directional preponderance of optokinetic nystagmus may be observed in patients with an unilateral labyrinthine lesion. In these patients the vestibular imbalance not only causes spontaneous nystagmus but also biases optokinetic nystagmus (OKN) (Ohm, 1932, & Mittermaier, 1939, Suzuki & Komatsu, 1962, Guttich & Okamoto, 1964, Coats,

The observations reported in the literature suggest an enhancement of OKN slow phase velocity if spontaneous nystagmus beats in the same direction as the OKN saccades, and a depression if it is in the reverse direction. Except for a few cases reported by Guttich & Okamoto (1964) there are, however, no quantitative data about this interaction. For the clinical diagnosis it is important to differentiate between directional preponderances of OKN due to an unilateral brain stem lesion and an exclusively peripheral labyrinthine defect. A concomitant brain stem lesion cannot be excluded if only depression of OKN-SPV occurs. The preponderance may

be recognized as "benign" and of peripheral vestibular origin if OKN is both depressed and enhanced by the vestibular imbalance. We therefore consider it useful to present briefly both computer evaluations of OKN slow phase velocity (SPV) influenced by labyrinthine lesions and a quantification of the directional preponderance with respect to the SPV of spontaneous nystagmus.

METHODS

Patients

Six female and 2 male patients, 19 to 52 years old, volunteered for this clinical series of experiments. All of them suffered from an acute unilateral peripheral vestibular lesion and initially complained of severe vertigo, gait disturbance, oscillopsia, and motion sickness. Four of the lesions were on the right side and four on the left. Each was interpreted to be either traumatic, vascular, or inflammatory in origin. The diagnosis was based on a careful neurological and electronystagmographical examination including the demonstration of unilateral labyrinthine underexcitability by caloric testing (Fitzgerald & Hall-

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pike 1942) Two patients could be investigated repeatedly during the course of their recovery (be it by central compensation—Bechterew, 1883, Schaefer & Meyer, 1975—or by restitution of labyrinthine function) None of the patients gave indications of having additional brain stem, cerebellar, or cortical disturbances

Apparatus recordings of eye movements, and data analysis

The experiments were performed using a modified version of the Tonnes Electro-nystagmography System This consisted of a servocontrolled, motor driven rotary chair located in front of a large segment of a cylindrical screen the longitudinal axis of which was centred with respect to the subject's head The subjects sat in the upholstered chair fitted with a head support so that the screen with its 79.5 cm radius of curvature subtended a visual angle of about 100° vertical and 140° horizontal extent An optokinetic stimulator was used to continuously project a regular pattern of vertically oriented black and white stripes each 7.5° wide These moved horizontally at constant angular velocities of 30°, 60°, 100°, and 120°/sec respectively to either the left or right The optokinetic stimulus field could be restricted in its horizontal dimensions to a visual angle of 30° by partial covering of the stimulator's small field stimulation

Horizontal eye movements were recorded using silver-silver chloride surface electrodes (Beckmann) placed over the outer canthus of each eye After d.c. amplification the signals were stored on a 7 channel FM tape recorder (Bell and Howell VR 3200) for subsequent computer analysis

Calibrations of eye movements were taken from voluntary saccades of increasing visual angle (15°, 20°, 30°, and 40° to the left and right) which were separately least mean squares fitted for right and left calibrations by two cubic curves The signals of eye movements recorded on tape were analog to digital converted and stored on digital computer disks

(sampling interval 10 msec) The recordings were analysed using a modified computer program adapted from MITNYS II (Allum et al 1977) on an IBM 1130 computer This program automatically computed for every 10 msec the slow phase velocity (SPV) of optokinetic nystagmus, which was averaged over 10 sec intervals Where a saccade occurred the saccade's amplitude, duration and maximum average velocity were also analysed Because of the limited storage capacity of the computer only one period of optokinetic nystagmus could be analysed at one time Curves displayed on a computer plotter presented the original nystagmogram together with the actual slow phase velocity as well as occurrence and duration of fast phases Computer printouts contained the 4.8 sec slow phase velocity averages

For quantitative evaluation of the directional preponderance of optokinetic nystagmus the interval with the lowest coefficient of variation (standard deviation divided by SPV) within the recorded period of 10 sec was first determined Using these intervals the difference in OkN slow phase velocity between two horizontal directions was then expressed as a percentage of the faster OkN

Experimental procedure

After calibration of horizontal eye movements spontaneous vestibular nystagmus was recorded first with the eyes closed The intensity of spontaneous nystagmus is dependent on vigilance subjects were asked to perform mental arithmetic Spontaneous nystagmus was again recorded at the end of each experiment

Directional preponderance of optokinetic nystagmus

Optokinetic nystagmus (OkN) was then tested by asking subjects to follow the moving stripe pattern at 20°/sec Two 20-sec periods of optokinetic nystagmus were recorded for each constant stimulus speed (30°, 60°, 100° and 120°) in each horizontal stimulus direction The

EF 28 J traumatic right labyrinthine lesion

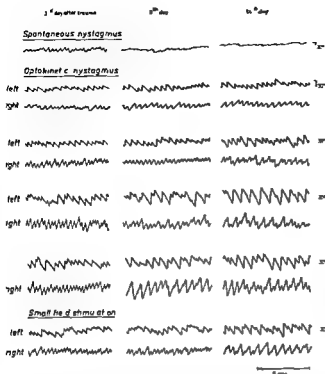


Fig 1 Original recordings of the horizontal component of spontaneous nystagmus and optokinetic nystagmus 3, 9 and 14 days after a right traumatic labyrinthine lesion. Directional preponderance of OKN toward the side of spontaneous nystagmus increases with increasing stimulus speed and progressively vanishes with compensation for the vestibular imbalance.

parated by 30–60 sec relaxation periods. ally OKN to the left and to the right was ed with the small field of stimulation at an ular velocity of 60°/sec.

RESULTS

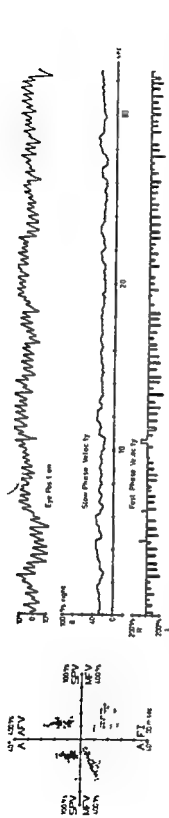
Spontaneous vestibular nystagmus

patients investigated exhibited spontaneous nystagmus. This was invariably directed towards the side opposite to the lesioned labyrinth. The average slow phase velocity (SPV) of spontaneous nystagmus over a registration period of 20 sec ranged from 1° to 32.7°/sec. The 2 patients who were repeatedly studied during recovery showed a progressive decrease in SPV of spontaneous nystagmus. Frequently the SPV of spontaneous vestibular nystagmus exhibited a considerable variance within and between several trials in a subject. This happened despite the attempt to maintain vigilance by asking the sub-

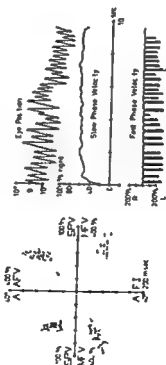
jects to perform mental arithmetic. For the evaluation of the consequences of vestibular imbalance on optokinetic nystagmus the 11 recordings from 8 patients were arbitrarily divided into two groups of spontaneous nystagmus intensity. The dividing criterion was whether the average slow phase velocity was above or below 12°/sec. The average values of group 1 were 2.5°, 7.8°, 11.1°, 11.2°, 11.3°, 11.5°, 11.5°/sec. Those of group 2 were 12.7°, 15.0°, 22.9°, 32.7°/sec.

Optokinetic nystagmus

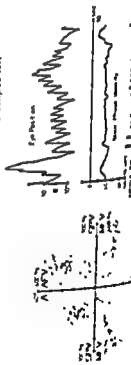
All patients tested consistently revealed a directional preponderance of OKN. This was always toward the direction of spontaneous vestibular nystagmus and occasionally reached 65–75% in cases with a severe vestibular imbalance. The original recordings in Fig 1 and the computer traces of slow phase velocity in Fig 2 may serve as an illustration. Their evaluation together with the data



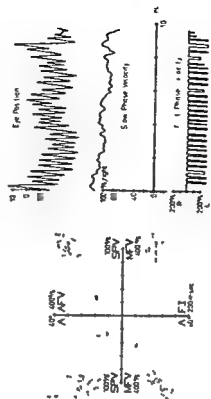
OKN 60/sec stripes right



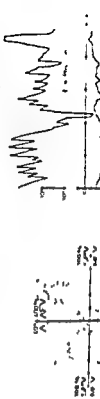
OKN 60/sec stripes left

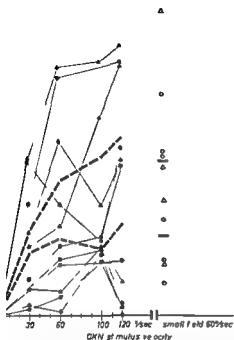


OKN 120/sec stripes right



OKN 120/sec stripes left





Percentual OKN preponderance (ordinate) in relation to stimulus velocity (abscissa). Individual data of Δ with spontaneous nystagmus—SPV exceeding 1 (triangles) and those with smaller spontaneous nystagmus (dots). Dashed lines show the averages of the two groups for each stimulus velocity. Corresponding data for field stimulation on the right.

presented in Fig. 3 allow of the following additional statements:

(a) For a given spontaneous nystagmus the directional preponderance of OKN slow phase velocity tends to increase at higher stimulus speeds. The preponderance may be absent or weak at lower stripe velocities of 30°–60°/sec. Using a small field of 30° horizontal extension of optokinetic stimulation, the percentual difference between the two directions becomes almost as large at 60°/sec as with full field

stimulation at 100° or 120°/sec, although the absolute difference is not as great. This is illustrated by the averages presented in Fig. 3. In four cases with moderate spontaneous nystagmus (slow phase velocity) the directional preponderance was so weak that it did not fall outside the range of normal variation (15%–22%).

(b) The percentual difference of OKN slow phase velocity to the right and to the left increases on average with the mean slow phase velocity of spontaneous nystagmus. This is documented in Fig. 3, by the difference between the two intensity groups. For group 2, presenting the more vivid spontaneous nystagmus, the difference reached a mean of 40% at 120°/sec stimulus speed, whereas the corresponding values in group 1 hardly exceeded 20%. There is however no close correlation between vestibular nystagmus intensity and the degree of OKN directional preponderance, a fact that hampers diagnostic evaluation in individual patients.

OKN directional preponderance during compensation for a peripheral labyrinthine lesion

The difference in OKN–SPV generally decreases with decreasing SPV of spontaneous nystagmus during recovery from a peripheral labyrinthine lesion. So far, only the percentual differences of right and left OKN were discussed in cases of spontaneous nystagmus. For the clinician and the physiologist, however, it is of great interest to know whether this difference is due to a facilitation of OKN to the side opposite to the lesion, or an inhibition of OKN to the same side, or both. If it were due to inhibition alone, it would be very difficult for the clinician to decide whether the OKN diminution toward the side of the lesion was due to an additional brain stem lesion or not. From the absolute data it is difficult to answer, since, among the cases studied, none exhibited an OKN–SPV exceeding stimulus velocity, although initially they had abnormally high values of SPV. The fact that in a

2. Example of a computer evaluation of nystagmus. This plot contains the original eye movements, a computer evaluation of slow phase velocity (SPV) and the times of fast phases. Data depicted in cross-coordinates on the left relate SPV to amplitude (A) and age velocity (AFV) of fast phases as well as maximum phase velocity (MFV) to amplitude (A) and interval of fast phases (FI).

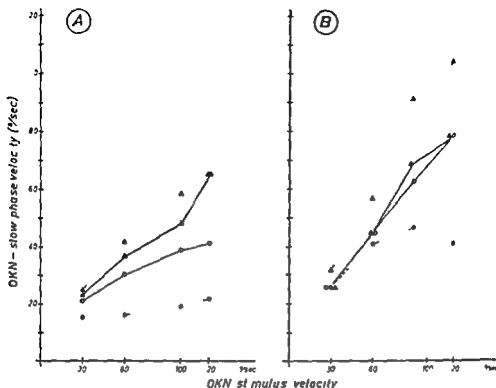


Fig. 4. OKN slow phase velocity (ordinate) in relation to stimulus velocity for 2 individual subjects (A, B). The initial recordings after labyrinthine lesion (Δ, ●) show a significantly greater difference in SPV than those obtained

11 days later (Δ, ○) when spontaneous nystagmus had increased to one third. Data indicate a transient depression of OKN.

In some instances slow phase velocities of OKN reached $110^{\circ}/\text{sec}$ at $120^{\circ}/\text{sec}$ stimulation velocity when OKN was toward the direction of spontaneous nystagmus may indicate facilitation. The exceptionally low slow phase velocities of OKN opposite in direction to spontaneous nystagmus (i.e. $40^{\circ}/\text{sec}$ at stimulus velocities of $120^{\circ}/\text{sec}$) certainly prove inhibition. A stronger argument for the occurrence of both facilitation and inhibition of OKN is given by the results of repeated measurements during the course of recovery from or central compensation for a peripheral labyrinthine lesion. As can be seen from Figs. 1 and 4, slow phase velocity of enhanced OKN decreases with the diminution of vestibular imbalance as judged by spontaneous nystagmus-SPV, while SPV of the depressed OKN increases. It may consequently be assumed that the basically normal performance of the optokinetic system was biased both ways by the vestibular imbalance.

COMMENT

The results substantiate the old idea that vestibular imbalance may enhance or depress optokinetic nystagmus (Ogata, Jung & Muttermaier 1939; Suzuki & Tsukahara 1962; Guttich & Okamoto 1968). The data agree with observations in humans (Koenig et al. 1978) who show a variable gain of a transient vestibular effect dependent upon optokinetic stimulus velocity. The vestibular influence (OKN preponderance) is found to increase with increasing optokinetic pattern velocity and with the strength of the imbalance both for the mutually exclusive conditions of facilitation and depression. The depressing combination of the two interactions is thus not just purely subtractive but a feed forward control of the vestibular compensation (involved before the two are combined). It may be hypothesized that gain control is performed by the

ular flocculus receiving visual input (Hess & Alley, 1974). It seems to involve an additional individual component, since the relative correlation between spontaneous nystagmus intensity and the consequent directional preponderance of OKN is rather variable between individuals. Some other mechanism must be responsible for the bidirectional preponderance of OKN-SPV following bilateral labyrinthine lesions (Zee et al., 1976).

Directional preponderance of optokinetic nystagmus in the direction of spontaneous nystagmus is a frequent observation in patients with acute peripheral vestibular lesions. Directional preponderance, however, does not always exceed the range of preferences observed in normals. Depending on the stimulus condition the normal range is between 15% and 22% (Dichgans et al., 1974).

A combined lesion of the homolateral cerebellar and reticular formation may cause a similar directional preponderance but this will only lead to a diminution of OKN to the side opposite the lesion and not to an enhancement to the opposite side. A comparison between the recording performed in the acute phase and a second one performed 2 weeks later indicates the vestibular origin of an OKN preponderance by exhibiting a reduction of the enhancement along with the compensation for the vestibular imbalance. The best estimate of a possible OKN preponderance, in absolute and relative terms, is obtained by optokinetic stimulation either at rest or with an ample stimulus or with a small stimulus (Dichgans et al., 1974). Usually fast SPV and high frequency of OKN toward the side of spontaneous nystagmus alone suggest that the OKN preponderance is solely to vestibular imbalance and not to a brainstem lesion. OKN in patients suffering from a labyrinthine lesion is not purely visually induced but must be correctly termed *vestibularly biased OKN*.

In this context one may recall that gaze nystagmus in the direction of spontaneous nystagmus may be another consequence of peripheral

vestibular lesion and not due to involvement of the brainstem.

ZUSAMMENFASSUNG

Vestibuläre Tonusdifferenzen nach akuten einseitigen Labyrinthläsionen führen zu einer richtungsbestimmten Beeinflussung der Geschwindigkeit langsamer Phasen des optokinetischen Nystagmus (OKN). Dies wurde bei acht Patienten zum Teil wiederholt quantitativ untersucht. Alle zeigten ein Richtungsüberwiegen des OKN in Richtung des Spontan-nystagmus, das auf Bahnung des ipsiversiven und Hemmung des kontraversiven OKN beruht. Das Richtungsüberwiegen nahm im Mittel mit der Intensität des Spontan-nystagmus zu und mit dessen Kompensation ab. Es erreichte maximal 70%. Eine Abgrenzung gegen optokinetische Störungen bei Hirnstammläsionen ist möglich.

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VERTIGO AS REFLECTED BY THE NYSTAGMOGRAM

A Clinical Analysis

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Efforts to evaluate findings in the nystagmogram made in a material of 338 vertiginous and dizzy patients. A spontaneous nystagmus, a positional nystagmus as well as a difference in caloric reactivity are of little value for revealing peripheral lesions. On the other hand, central disturbances are frequently revealed by inability to track a moving optoelectric target in an irregular or a saccadic pattern, by persistence of vestibular nystagmus in light, by persistence of nystagmus at eye-closure, by an increase in spontaneous nystagmus on eye-closure or by dysrhythmic nystagmus in caloric tests. Cases of functional vertigo seem to come from other sources by their spontaneous nystagmus (when present) or by persistence of caloric nystagmus on eye-closure.

In the preceding papers a material of 338 vertiginous and dizzy patients was analysed with respect to the character of perceptions (Henriksson et al., 1976a), to duration of perceptions (Henriksson et al., 1976b) as well as to the significance of functional symptoms (Afzelius et al., 1977). In the present paper these findings will be related to the nystagmographic results.

In spite of excellent works on nystagmography (Aschan et al., 1956; Jongkees & Philips, 1964; Megighian, 1959) many positive findings are not entirely understood and their clinical interpretation is still doubtful. The aim of the present paper is therefore to deepen our understanding of these findings by looking for possible correlations between various objective findings and also to correlate these findings to perceptions and symptoms. Access to data from an analysis of 338 patients (reported in our previous papers) with data added from complete routine nystagmograms forms the basis for our contribution.

MATERIAL AND METHODS

338 patients consulting our "Dizzy clinic" were carefully investigated, with anamnesis, otological examination as well as nystagmography, as described in previous papers.

The nystagmographic examination comprised the following procedures, previously comprehensively accounted for by one of us (Henriksson et al., 1967; Henriksson et al., 1972).

I Tracking test

In this test the patient was trying to follow with his eyes a target moving at constant angular velocity on a circular screen one metre in front of him. Pathological findings were: 1, Irregular tracking patterns; 2, Saccadic tracking pattern.

II Spontaneous eye movements

Test in darkness for spontaneous eye movements with open and closed eyes for: 1, Spontaneous horizontal nystagmus; 2, Undulating eye movements; 3, Effect of eye-closure on spontaneous nystagmus.

III Caloric nystagmus responses (open eyes in darkness)

This test was undertaken for: 1, Difference in caloric reactivity; 2, Persistence of caloric nystagmus at eye closure; 3, Dysrhythmia.

A test for gaze- and positional nystagmus was also made.

Information from the questionnaire and from the nystagmogram was fed into a com-

- VJ (ed H H Kornhuber) vol 2 p 451 Springer, Berlin Heidelberg New York
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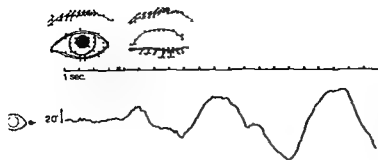


Fig 3 Undulating eye movements behind closed eyes

ints of the target. In between these the eyes moved slower than the target could in some cases be nearly or completely at a standstill. This pattern of eye movement gave the recording a very characteristic appearance (Fig 1E-F).

Prevalence. Thirteen patients had a slight pattern and 7 had pronounced sac-

Correlations. Patients with gaze nystagmus rarely had saccades in the tracking tests compared with 1% among the other patients (5% significance). Further, the saccades were also significantly often combined with inhibition of vestibular nystagmus (high ocular fixation index according to Leuz & Ledoux, 1969). All 5 serious cases of acoustic neuromas, 3 cases of multiple sclerosis in this material were found among patients with pronounced saccades.

Interpretation. Gaze nystagmus and saccades are positively correlated. They may be similar but possibly not always identical variants of eye motor coordinating structures. The clinical importance of pronounced saccades, almost proving serious central disease, must here be stressed. It must be mentioned, however, that also only moderate use of barbiturates or other sedatives may cause this characteristic tracking pattern.

II Spontaneous Eye Movements

Spontaneous horizontal nystagmus

Appearance. Spontaneous nystagmus may be detected when not complicated by simul-

taneous eye blinks. The derived recording technique frequently helped by reflecting the fast component as a conspicuous spike.

Frequency. 72 patients had spontaneous nystagmus (open eyes in darkness). Nystagmus beats appearing only behind closed eyes were not included as they may well be looked upon as normal findings.

Correlations. Only one significant correlation between existence of spontaneous nystagmus and other signs or symptoms was found. Patients with spontaneous nystagmus had significantly fewer undulating eye movements behind closed eyes than other patients (3% against 14% among other patients).

Interpretation. The lack of correlation to other signs and symptoms is interesting. Nystagmus is then—although it represents an objective background for vertigo—of little value for reaching a diagnosis or determining whether a central or a peripheral source of vertigo may be expected. The negative correlation to undulating eye movements is explained by the fact that nystagmus decreases in sleepiness (Collins & Guedry, 1962) and by the fact that undulations are frequent in sleepy patients (Hamersma, 1957).

2 Undulating eye movements

Appearance. Undulating eye movements were frequently of large amplitudes and with a frequency of 0.2–0.5 periods/second (Fig. 3).

Frequency. Undulating eye movements behind closed eyes were observed in 38 patients gazing straight ahead and in another 29 pa-

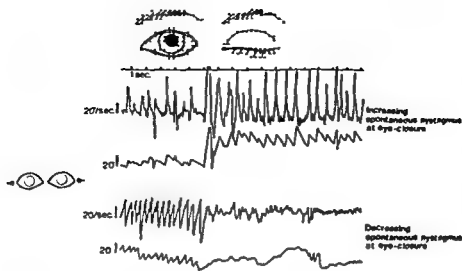


Fig. 4. Increasing and decreasing spontaneous nystagmus at eye-closure.

tients there were such movements only when gazing to the right or to the left.

Correlations. Apart from the above mentioned negative correlation to spontaneous nystagmus, patients in this group differed from other patients only with respect to certain expressions of social adaptation. They were thus more frequently divorced, 16% against 5% for other patients, their conjugal relations were less good, 8% against 2%, and they regarded their economy as poor, 21% compared with 11% among other patients.

Interpretation. The negative correlation between spontaneous nystagmus and undulating eye movements was discussed above. There is a general agreement that these eye move-

ments express sleepiness (Aschan et al. 1957, Aschan, 1967, Clement 1970, Elmqvist et al. 1957). Sometimes in patients with disturbances undulations are mere expressions of a pathological sleepiness (Elmqvist).

The relation between presence of undulations and a poor social adaptation is difficult to interpret. Possibly this is a coincidental significance. However, it raises the question whether the sleepiness denoted here by the presence of undulations also indicates a certain social environment.

3. Effect of eye closure on spontaneous nystagmus

Appearance. Spontaneous nystagmus is frequently rather heavily modified by eye closure (Fig. 4).



Fig. 5. Number of early functional symptoms in % in patients with increasing (I.N.) and decreasing (D.N.) spontaneous nystagmus upon eye-closure and in patients without spontaneous nystagmus (remain. pat.).

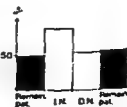


Fig. 6. Number of late functional symptoms in % in patients with increasing (I.N.) and decreasing (D.N.) spontaneous nystagmus upon eye-closure and in patients without spontaneous nystagmus (remain. pat.).

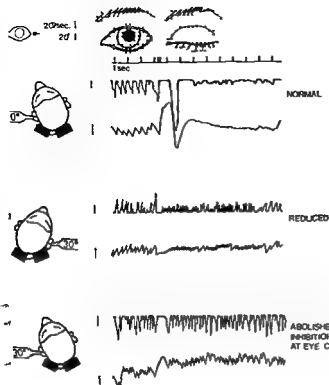


Fig 7 Various degrees of inhibition of calorically induced nystagmus by eye-closure

frequency 72 patients in the material had spontaneous nystagmus. In 34 cases of these there was an increase in nystagmus and in 21 cases (21%) there was a decrease in nystagmus upon eye closure.

Relations. Patients who had a decrease in spontaneous nystagmus upon eye closure had relatively few early functional symptoms (significance almost at a 5% level, see Fig 5). Spontaneous nystagmus increased upon eye closure was found in patients with many and especially late functional symptoms (significance at a 1% level) (Fig 6).

Interpretation. The intensity of nystagmus upon eye closure may thus be assumed to reflect nervousness in the patient. This correlation may seem difficult to explain. It is well known, however, that sleepiness reduces nystagmus (Collins & Guedry 1962). It is also known that eye closure enhances sleepiness. This is clearly reflected by the increase of alpha rhythm in the EEG. The decrease of nystagmus upon eye closure in normals could

then be an effect of sleepiness provoked by eye closure. In nervous patients suffering from anxiety and irritability an increased alertness may be expected. In these patients eye closure may then *not* convey the normal decrease in alertness allowing the nystagmus to stay unaffected by eye closure.

In this behaviour spontaneous nystagmus upon eye closure seems to reflect personality and may help to differentiate between func-

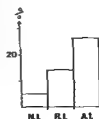


Fig 8 Number of patients in each group with persistent nystagmus in light, distributed in the groups with different inhibition of nystagmus upon eye-closure. NI = Normal, RI = Reduced and AI = Abolished inhibition by eye-closure.



Fig 9 Number of patients in 1% with undulating eye movements upon eye closure distributed in the different groups of inhibition of nystagmus upon eye-closure. NI = Normal RI = Reduced and AI = Abolished inhibition by eye-closure

tional and other sources of vertigo and dizziness

III Information by Caloric Nystagmus Responses

1 Difference in caloric reactivity

Appearance The intensity of the caloric response was measured by maximum speed of the slow component. A difference between right and left ear exceeding 20% was, according to Jongkees & Philipszoon (1964), regarded as significant.

Frequency 87 patients showed a difference in caloric response exceeding 20%.

Correlations These 87 patients did not differ much with respect to other signs and symptoms from patients with equal caloric responses. There was no correlation to one-sided loss of hearing, to a spinning sensation outside head, nor to other signs or symptoms indicating a labyrinthine disorder. On the other hand, of the 17 confirmed Meniere cases 59% showed a difference in reactivity against 24% among other patients.

Interpretation Thus even if unequal caloric responses are frequently found among Meniere cases, the solitary finding of a difference in caloric reactivity does not significantly indicate this disease. It may then be concluded that clinical information derived from the solitary finding of a difference in caloric response so often assiduously looked for, must be regarded as rather overestimated.

2 Persistence of caloric nystagmus at eye closure

Appearance Normally vestibular nystagmus is distinctly inhibited by eye-closure (Strom 1973). However, this inhibition of caloric nystagmus seems, however, tends to be moderate or even missing (Fig 7).

Frequency In most patients in this group this normal inhibition of nystagmus upon eye-closure was missing. In 104 patients there was a decrease—although quite moderate—in intensity. In 10 patients there was little or no change in nystagmus upon eye-closure.

Correlations Caloric nystagmus upon eye closure also persisted during fixation in light (Fig 8). It rarely appeared in patients with normal nystagmus (Fig 9).

Patients in this group with persistent nystagmus upon eye closure also comprised a large number of functional symptoms. It had been present even before the onset of vertigo (Fig 10). The difference in frequency from the group with normal inhibition of nystagmus upon eye closure was significant at a 1% level.



Fig 10 Number of early functional symptoms attributed in the different groups of inhibition of nystagmus upon eye-closure. NI = Normal RI = Reduced and AI = Abolished inhibition by eye-closure

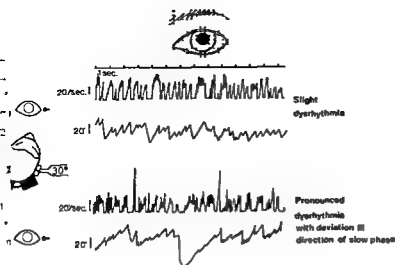


Fig 11 Different degrees of dysrhythmia and deviation of the eyes in direction of slow phase during caloric test in two different subjects

Interpretation: According to Demanez & Lix (1969) the persistence of caloric nystagmus in light indicates a central disturbance due to the positive correlation, persistent nystagmus at eye closure should also be an indicator of central disturbance.

The lack of undulations among these patients supports the assumption of some kind of abnormality among patients in this group. Such an abnormality seems to preserve the nystagmus by counteracting the sleepiness conveyed by eye closure by the same mechanism as discussed for spontaneous nystagmus upon eye closure. The assumption of a nervous arousal or tension is further supported by the finding of a large number of functional symptoms—nervousness and irritability. Along with these symptoms can be expected a tension forming the basis for the arousal. In this way the three correlations may be explained along the same lines. Anxious patients have a higher level of arousal making expressions of sleepiness, undulations rare and allowing nystagmus to persist in spite of eye closure.

Dysrhythmia in the caloric nystagmus response

Appearance: An adequate and practical definition of dysrhythmia seems still to be missing. The degree of dysrhythmia had then to be subjectively evaluated. Examples of slight and

pronounced dysrhythmia in the caloric test are presented in Fig 11.

Frequency: 11 patients had a slight and 8 a pronounced dysrhythmia in the caloric nystagmus response.

Correlations: Most patients in the pronounced dysrhythmia group were found in the highest age decade (71–80 years) (Fig 12). Patients with dysrhythmia in the caloric nystagmus response complained of a large number of early functional symptoms (Fig 13). The difference vis à vis other patients was significant at a 5% level. No differences were found with respect to late functional symptoms (Fig 14).

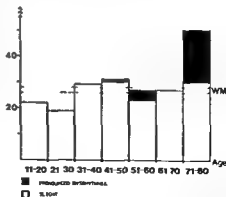


Fig 12 Number of patients in % with pronounced and slight dysrhythmia within the different decades. The two lines in the figure represent the two types of dysrhythmia in the whole material.



Fig 13 Number of early functional symptoms in patients with dysrhythmia (DR) and without dysrhythmia (remain pat)

Interpretation An assumption that dysrhythmia expresses disturbances in central integration essentially on a vascular basis (Henriksson et al., 1972) is supported by the fact that dysrhythmia was so frequent among the oldest patients. The clinical value of this, however, is diminished by the fact that also early functional symptoms seem to correlate to dysrhythmia.

COMMENT

Spontaneous nystagmus may be the sign most frequently looked for in the nystagmogram. This finding, although frequently met with in our recordings, presented unexpectedly few correlations to other signs and symptoms. Thus, nystagmus seems to appear just as frequently in peripheral as in central disorders.

The negative correlation between the presence of spontaneous nystagmus and presence of undulations has not been reported earlier and may be of no great clinical interest. It does confirm, however, the concept that the intensity of spontaneous nystagmus is expressing alertness and that the presence of undulations expresses sleepiness.

The difference in caloric reactivity between the two ears, a sign very assiduously sought by many otologists, showed no significant

relations, whether to unilateral benign vertigo lasting for 2-5 hours or to functional symptoms which might be expected. This indicates that the finding of a difference in caloric reactivity from clinical point of view may be somewhat overestimated earlier.

Thus, even if the otologist might be disappointed when looking for indicating a peripheral lesion, many of the nystagmograms seem to be of value in the evaluation of the cases by supporting the diagnosis.

A positive tracking test, although not specific, gives weighty support to a disturbance as being responsible for the vertigo. The regular but pathological movements of the tracking test replacing the normal flexible movements seem to be the most relevant finding in the whole graphic test battery. This sign of pathological saccades was present in all 5 series of the material.

Saccades instead of smooth fixations, gaze nystagmus and persistent nystagmus in light are all findings that can be interpreted as expressions of central disturbances. The obvious connection between pathological saccades and gaze nystagmus on the one hand and also between these and persistence of nystagmus in light on the other, supports this assumption.

The various objective findings in the nystagmogram connected with early and late functional symptoms also seemed to be of

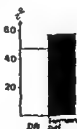


Fig 14 Number of late functional symptoms in patients with dysrhythmia (DR) and without dysrhythmia (remain pat)

In patients with early functional nystagmus the caloric nystagmus was dys-ic and also persisted in spite of eye closure. The vertigo in these cases was very resistant against treatment.

Patients with late functional symptoms, late-onset nystagmus when present tended to ease at eye-closure. The prognosis with respect to vertigo in these cases was relatively

different. Differences between the two different types of symptoms connected with early-onset functional symptoms also indicated different pathology in such cases.

To conclude, even if the nystagmogram does not often directly inform about labyrinthine disorder, a lack of signs indicating central disturbances may support such a diagnosis. However, a long row of signs in the nystagmogram would seem to reveal central disturbances possibly related to vertigo.

ZUSAMMENFASSUNG

Untersuchung betraf 338 Patienten mit Schwindelgelegenheit. Es darauf ankam, nystagmische Befunde zu interpretieren. Um periphere Erkrankungen des Vestibularsystems zu erfassen, sind Symptome wie Spontan- und Latenznystagmus und Unterschiede in der kalorischen Erregbarkeit von begrenztem Interesse. Zentrale Störungen kann man dagegen erfassen, indem man einen Test zur Blickfolge eines beweglichen Zieles mit dem Erscheinen einer irregulären oder Saccaden-ähnlichen Nystagmusreaktion oder ein Bestehen einer Nyssreaktion im Licht und beim Augenschließen oder eine Zunahme des Spontan- und Latenznystagmus beim Augenschließen oder bei einer dysrhythmischen Reaktion im Licht. Test, Patient mit psychischem Schwindel unterscheiden sich von Schwindelpatienten mit anderen Ursprungs durch ihre Zunahme des Spontan- und Latenznystagmus (wenn vorhanden) oder bei dem Bestehen eines latenznystagmischen Nystagmus beim Augenschließen.

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NASAL MUCOCILIARY FUNCTION DURING PENICILLIN TREATMENT

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Abstract Nasal mucociliary function was studied in 21 healthy subjects before, during and after penicillin administration. No change in the mucociliary function occurred during penicillin treatment. Nor was any difference observed between men and women or between smokers and non smokers. On the other hand the measurements revealed a significantly smaller intra individual than inter individual variation. As the epithelia of nasal cavity and Eustachian tube are morphologically of the same type, it is concluded that the increased incidence of serous otitis media which has been observed after the introduction of penicillin in the treatment of acute otitis media can scarcely be due to an inhibitory effect of penicillin on the mucociliary function of the Eustachian tube.

In-vitro studies have shown that penicillin exerts an inhibitory action on the ciliary function. This was demonstrated by Proetz as early as 1945 in ciliated respiratory epithelium removed from the tracheal mucosa of rabbits. The effect was related to the concentration. Thus, a solution of 5000 I U of penicillin per ml reduced the ciliary movements to one-half within a few minutes, whereas a solution of 125 I U per ml was without any effect. Strong & Tonkin (1951) who used human mucosa from the maxillary sinus removed during operation for sinusitis demonstrated that the ciliary movements ceased after 2 minutes when the mucosa was rinsed in a penicillin solution containing 300 000 I U per ml, and that the effect was reversible. In recent in-vitro studies on the mucociliary function of the rabbit trachea, Toremalm et al (1977) likewise demonstrated that a penicillin solution exerted a pronounced inhibitory effect on the ciliary activity resulting in ciliostasis within 1-2 hours, but they did not report

the penicillin concentration used. On the other hand, a 10% gentamycin solution had no effect on the ciliary activity.

If this effect of penicillin on the ciliary epithelium of the Eustachian tube is also valid in vivo, this might possibly explain the increased incidence of serous otitis media which has been observed during the last years and which seems often to have occurred after preceding penicillin therapy for acute otitis media (Kokko, 1974).

The purpose of the present study was to determine the clearance in healthy subjects and to investigate whether penicillin in therapeutic doses exerts an inhibitory effect on the mucociliary function in vivo. It must be assumed that the cilia of the mucosae of the Eustachian tube and the nasal cavity are identical, since the epithelium of the Eustachian tube is morphologically of the same type as that of the respiratory tract (Sadé, 1966; Hentzer, 1973).

MATERIAL AND METHOD

The mucociliary function was studied by the saccharin/sky blue technique described by Andersen et al (1974a). A blue saccharin particle, 0.5-1.0 mm in diameter, was placed on the superior surface of the soft palate, about 0.5 cm behind its anterior end, and 4-5 cm from the apex of the nose. The subject was then instructed to swallow once every 30 seconds, and the time lapse until the particle was no longer visible was recorded. The strong sweet taste is recorded. The passage of the particle is verified by palpation of the mucosa of the nasopharynx.

I Nasal clearance before during and after administration of penicillin

Nasal clearance relative to penicillin administration											
ex	Age (yrs)	Before (min)			During (min)			After (min)			Serum conc (μ g/ml)
	27	7.5	7.0	10.0	10.5	9.0	9.0	8.5	11.0	8.0	6.9
	28	14.0	8.5	17.0	8.0	15.0	6.0	11.0	6.0	9.0	10.2
	24	11.0	11.0	13.0	10.0	8.5	9.5	11.0	10.0	12.0	7.8
	24	7.5	6.5	8.0	14.0	14.0	12.5	12.0	17.0	11.5	7.6
	25	-	7.5	9.0	7.5	7.0	-	7.0	10.0	14.0	17.2
	26	13.0	10.0	11.0	9.0	7.5	9.0	9.0	10.0	10.0	9.3
	28	-	10.0	9.0	12.0	9.5	7.5	9.0	11.0	9.5	8.9
	27	12.0	10.0	14.0	11.0	8.0	-	14.0	9.0	10.0	7.2
	24	18.0	7.0	20.0	10.0	11.5	7.5	8.5	18.5	9.5	8.9
	25	8.5	6.0	6.5	6.5	8.5	9.5	8.0	6.5	11.0	21.5
	26	8.0	9.5	9.5	7.0	8.0	6.0	7.5	8.5	-	18.2
	24	8.0	7.5	4.0	7.0	7.0	7.0	7.5	7.5	6.5	8.5
	24	15.1	11.0	10.0	12.0	11.5	11.0	9.0	-	7.0	15.0
	26	17.5	9.5	9.0	8.0	12.0	9.0	-	8.0	6.0	14.5
	23	8.5	9.0	8.0	7.5	7.0	7.5	7.0	8.0	8.5	4.7
	24	10.5	12.0	11.0	10.5	7.5	9.5	9.0	11.0	7.5	14.6
	27	11.5	9.0	11.0	11.0	10.5	9.5	12.0	9.5	10.0	28.3
	25	-	8.0	9.5	9.5	8.5	8.5	7.5	10.0	8.5	4.9
	21	7.0	8.0	13.0	9.5	10.5	13.5	11.0	9.0	7.0	11.2
	19	18.0	12.0	11.5	17.5	12.0	16.0	9.0	10.0	9.5	10.3
	37	8.0	8.5	6.0	7.5	6.5	8.0	9.5	7.5	7.5	19.5
e		10.1 min			9.5 min			9.3 min			12.2

e of 181 measurements 9.6 min

e for men 9.4 min for women 9.9 min

e for smokers 9.9 min for non smokers 9.5 min

studies 7 were unsuccessful () one not performed () nos 1 6 8 and 20 excluded (see text)

y simple and easy method for the measurement of mucociliary nasal clearance. As by Andersen et al (1974a) there is a significant correlation between this method and the more complicated technique with active particles.

The subjects studied were 25 healthy medical students and nurses, of whom none had any respiratory tract infection during the preceding 3 weeks. Before each measurement the subject concerned was acclimatized by staying in a stable environment for at least 1 hour. Our studies extended over the period from March 1 to May 5 1977. Twice during the experimental period the temperature and relative humidity in our laboratory were recorded continuously for a week. The room temperature was a constant 21–23°C and the relative humidity of the air was 30–35%. Studied reported by Andersen et al (1972, 1974b) and Bang et al (1967) have shown that changes

in temperature and relative humidity of the order just mentioned do not have any effect on human nasal mucociliary function.

Each subject was studied on nine occasions, distributed in three series, viz (1) measurements were performed on three occasions before the administration of penicillin, (2) on three consecutive days during penicillin treatment, and (3) three times after this treatment. The penicillin was given by mouth in the form of three tablets, each of 1 million IU of phenoxymethylpenicillin (Fenoxillin[®], Novo) in the morning and evening for 3 days. The measurements of nasal clearance were performed 1½ hours after the morning dose and ½–1 hour after the presumed maximum serum concentration (Bergan et al, 1975). On the second day of penicillin treatment the serum concentration of penicillin was determined simultaneously with the clearance measurement.

RESULTS

Of the 25 subjects, four (nos 1, 6, 8 and 20) were excluded because of a nasal clearance time of more than 20 minutes on two consecutive measurements in the first series. Subject no 1 had a few nasal crusts, but otherwise no discomfort. No 6 had had sinusitis 6 weeks previously, and swelling and redness of the conchae were still present. Nos 8 and 20 were objectively normal and had no discomfort. The remaining 21 subjects were 11 men and 10 women whose ages ranged from 19 to 37 (average 25.4) years. Six subjects (nos 2, 3, 4, 11, 17 and 22) were smokers, stating that their average consumption of tobacco was 4 cigarettes daily.

The average nasal clearance time was 10.1 min before, 9.5 min during, and 9.3 min after the administration of penicillin. These values are not significantly different. Nor was any difference found between men and women or between smokers and non-smokers, but the intra-individual difference of the measurements was significantly smaller than the inter-individual one ($p < 0.05$).

The average serum concentration of penicillin was 12.2 µg/ml (range, 4.7–28.3 µg/ml). The penicillin treatment resulted in diarrhoea in 6 subjects (28.6%), in one (no 11) so severe that penicillin had to be withdrawn after 2 days.

CONCLUSION

Our study shows that penicillin administered orally to healthy subjects even in relatively large therapeutic doses does not have any effect on nasal mucociliary function. It is therefore assumed that penicillin does not have any effect on the ciliary function of the Eustachian tube either because its mucosa morphologically is of the same type as that of the nasal cavity. The increased incidence of serous otitis media observed after the introduction of penicillin for the treatment of acute otitis media can therefore suitably be ex-

plained by an inhibitory effect on the ciliary function of the Eustachian tube.

ZUSAMMENFASSUNG

Die mukoziliare Funktion in der Nase und nach Penicillinbehandlung bei 21 Personen untersucht. Keine Änderung der Funktion wurde während der Penicillintherapie beobachtet. Auch zeigte sich kein Unterschied zwischen Männern und Frauen oder zwischen Nichtrauchern. Dagegen zeigten die Ergebnisse, daß die intra-individuelle Variation kleiner war als die inter-individuelle. Die der Nasenhöhle und der Ohrtrompete denselben Typ ist, wird konkludiert, daß die Einführung von Penicillin in der Behandlung der Mittelohrentzündung festgestellt werden kann einen hemmenden Effekt des Penicillin auf die mukoziliare Funktion in der Ohrtrompete zu haben.

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MUNOGLOBULINS IN NASAL SECRETIONS AND NASAL MUCOSA IN PERENNIAL RHINITIS

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Quantitative identification of albumin and immunoglobulin G, A, M and E in the nasal secretion and mucosa was done in 25 patients with perennial rhinitis and 25 normal subjects. By immunofluorescent microscopy plasma cells were identified by using FITC-conjugated globulin against IgG, IgA, IgM and IgE. The concentration of IgG and IgA in the nasal secretion and serum was significant and there was no difference between normal and patient groups. IgM was detected in nasal secretion in the patient group probably due to increased permeability of the mucosa. This explanation is supported by the fact that albumin was also found in a rather high concentration in the patient material. The presence of IgE-producing plasma cells was more frequently found in the patient group than in the control subjects and in those cases where presence of these cells was proved a high concentration of IgM in serum was shown, possibly indicating a generally increased IgM activity. This is in contrast to the case with IgE. Furthermore, increased IgE in nasal secretion and serum was found in the patient group. Identification of antigen specific IgE (RAST) in nasal secretion gave poorly reproducible and uncertain results which could not be correlated with the results from the analysis of serum.

During the past 10 years the role played by IgE in the pathogenesis of allergic diseases has been the subject of extensive research. The measurement of total IgE (RIST) and allergen specific IgE immunoglobulins (RAST) in serum has been of great importance in the differential diagnosis of asthma and atopic dermatitis (Johansson et al. 1972). The immunoglobulins in nasal secretions have also been studied in asthma, hayfever and polypoid rhinitis (Day et al. 1971; Younginger & Gleich 1971; Mygind et al. 1975) and the correlation between cutaneous, nasal and inhalation tests has been studied by Hourri et al. (1972). IgE-producing plasma cells were detected in

nasal mucosa of normal individuals for the first time by Tada & Ishizaka (1970), compared with the number of IgA- and IgG-producing plasma cells, however, they occur very sparsely (Mogi, 1975). No significant differences in the distribution of IgA, IgG and IgM in the nasal mucosa in normal and allergic patients have been found (Brandtzaeg et al., 1967), and the distribution of IgE-producing plasma cells in the nasal mucosa and polypoid rhinitis in atopic patients has not been significant (Pesak, 1971; Bass et al., 1974).

Perennial rhinitis presents considerable difficulties in differential diagnosis. With the methods of testing commonly used in allergology it is often not possible to identify a specific allergen, and these cases are often attributed to a vasomotor, unspecific instability. We have therefore found it helpful to examine the occurrence of immunoglobulins in nasal secretions in patients with perennial rhinitis, particularly by screening for allergen specific IgE antibodies, compared with the level in normal subjects. A fluorescent microscopic study has been done of immunoglobulin-producing plasma cells in biopsies from the inferior turbinate.

MATERIAL AND METHOD

Twenty-five patients with perennial rhinitis of at least 2 years' duration were included in the study. The history included running nose, sneezing and blockage but without seasonal variation.

Table 1 Albumin and immunoglobulin concentrations in serum and nasal secretion in normals (N-1 to N-15) and 25 patients (P-1 to P-25) with perennial rhinitis

	Serum					Nasal secretion				
	Alb (g/l)	IgG (U/ml)	IgA (U/ml)	IgM (U/ml)	IgE (U/ml)	Alb (g/l)	IgG (U/ml)	IgA (U/ml)	IgM (U/ml)	
N 1	45	126	133	192	70	42	47	135	46	
N 2	43	114	65	83	40	42	24	84	0	
N 3	46	88	78	109	100	42	15	84	0	
N 4	48	102	70	127	0	24	09	84	0	
N 5	38	90	83	66	0	51	12	60	0	
N 6	45	152	85	213	0	30	36	99	0	
N 7	41	127	106	37	0	33	21	84	0	
N 8	38	102	95	71	30	43	48	168	0	
N 9	42	176	133	50	0	45	45	129	0	
N 10	36	88	151	144	0	33	21	102	0	
N 11	47	109	111	81	120	30	27	144	0	
N 12	39	177	101	297	40	48	39	144	0	
N 13	40	123	119	207	0	33	42	120	0	
N 14	44	151	71	99	0	15	87	321	0	
N 15	38	102	103	126	30	03	09	87	0	
P 1	47	123	59	156	340	51	75	198	84	
P 2	44	102	67	189	160	27	24	120	0	
P 3	47	102	78	244	130	15	30	180	0	
P 4	41	113	125	97	250	114	324	414	118	
P 5	42	177	94	104	220	51	27	198	0	
P 6	43	90	67	156	60	78	21	99	0	
P 7	44	137	125	280	100	69	33	108	0	
P 8	41	102	67	207	800	48	15	81	0	
P 9	46	137	157	104	0	27	24	213	0	
	49	190	181	112	140	48	99	156	0	
	45	91	117	97	0	66	12	45	0	
	45	158	86	187	140	69	30	75	0	
	46	123	133	97	210	57	36	141	0	
	47	202	71	71	100	54	12	102	0	
P 15	44	151	23	99	160	39	68	231	0	
P 16	43	137	139	126	10	15	09	45	0	
P 17	43	158	101	140	0	60	12	112	0	
P 18	48	113	139	112	280	111	209	288	129	
P 19	45	113	94	120	170	42	15	111	0	
P 20	41	145	94	199	150	18	39	270	0	
P 21	48	179	0	213	10	66	105	27	159	
P 22	50	126	150	39	120	57	24	102	0	
P 23	47	114	106	101	4000	93	117	135	66	
P 24	50	114	114	90	40	45	30	63	0	
P 25	45	166	146	109	160	75	18	84	0	

Prior to inclusion in the trial a standard ENT examination was done including X ray of the nasal sinuses. Patients with polyp nasal crusting obstruction with active sinusitis and patients requiring treatment for asthma were excluded from the trial as also were individuals under 15 years of age.

The control subjects were 15 volunteers with no history of nasal disease or allergic manifestations.

Investigations for eosinophils in the nasal

secretion as well as prick test for allergen were done.

Examination of nasal secretion

The nasal secretion was taken on Whatman 41 with filter paper strips (Whatman 41) described by Lonn et al (1972) and et al (1975). Phosphate buffered saline (Coon's buffer, pH 7.1), so that the pH was approximately 7.2, and the osmotic factor was established according to the

Nasal Secretion

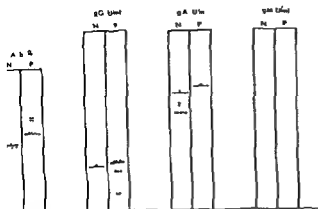


Fig 1 ■ agram showing albumin levels as well as IgG IgA and IgM in nasal secretion on N normal material P patient material

tion was done by centrifuging for 10 es at 15 G in a 5 ml plastic syringe with orated base fitting into a plastic centri glass. After passing through a Milipore about 2 ml clear eluate was obtained was discarded if there was a reaction to after testing with Hemastix test paper e level of albumin was measured by using modified electro-immunoassay technique ell 1966) and the amounts of IgG IgA IgM in serum and nasal secret on were used by a modified electro-immuno tech : by carbamylating the samples prior to rophoresis (Weeke 1968). Total IgE lev ere measured by the radioimmunosorbent uque (RIST) with Phadebas® IgE kit and gen specific IgE antibodies against 13 al ns were identified by using the Phadebas® T analysis system

nination of serum

centration of albumin IgG IgA IgM and as well as RAST screening were done in same way on serum from the patient ma l and the control subjects

unofluorescent investigation

ler local anaesthesia biopsies were taken 1 the inferior turbinate placed in AMES T compound and frozen to -20°C . Im iofluorescent microscopy was performed

as described by Crabbe et al (1965) using a direct technique in a $4\text{ }\mu\text{m}$ thick piece of tissue by conjugating with FITC marked rabbit antihuman gamma globulin (Behringwerke) di rected at IgG IgA IgM and IgE. After wash ing off of surplus antibodies the specimens were studied under a Leitz fluorescent micro- scope. The results were controlled by blocking tests with corresponding unmarked antisera. The blocks were fixed in paraffin wax and methylgreen-pyronin stained specimens were produced for assessment of the number of plasma cells in the biopsies

RESULTS

The levels of albumin and IgG IgA IgM and IgE in serum and in the nasal secretions of patients and controls are shown in Table I and Figs 1 and 2

The comparative mean values and in cases where the material could not be proved of normal distribution the medians are shown in Table II. From this it appears that contents of albumin IgM and IgE in the nasal secretions are significantly higher in the patient material than in the controls

In serum higher albumin and IgE levels were found in the patient group

Five patients showed a reaction to prick tests to various foods (fruit meat fish) as well

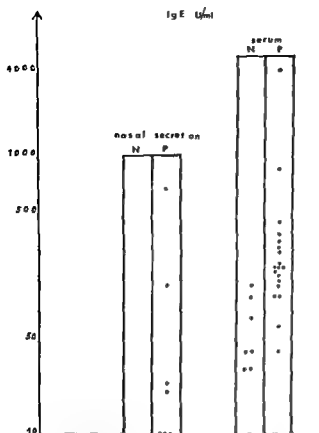


Fig. 2. Diagram showing levels of IgE in nasal secretion and serum. N=normal material, P=patient material.

to pets (dog, horse). Half of the patients did not show nasal eosinophilia and only a few showed eosinophilia to a marked degree.

No correlation between these findings and the immunoglobulin levels in serum and secretions was found.

RAST-screening to 13 different antigens in nasal secretions resulted in 10 proved positive reactions (in class 2). These results could not be related to the values obtained by RAST analysis in serum and no similarities between prick test and RAST analysis in serum and nasal secretions were found.

As the results were difficult to interpret, another investigation of the RAST in serum and secretions was done 6 months later in 1/4 of the patients. In these secretions values found were still low, but there was no reliable agreement with the previously found values.

In the biopsies a number of artefacts were found in the methylgreen-pyronin stain, which were due to the preparation technique. This did not permit any actual histological evaluation. The number of plasma cells was scanty and in some few specimens the presence of plasma cells could not be demonstrated.

The presence of IgG and IgA fluorescent plasma cells was not notable and did not appear equally in the control group and the patient group. IgM fluorescent plasma cells were seen in 7 patients, but only in one of the control subjects. IgE fluorescent cells were found rather frequently, i.e. in 9 patients and 4 controls. In nearly all instances they were found in isolation (Fig. 3) and only

Table II. Statistical evaluation of albumin and immunoglobulin concentrations in serum and nasal secretion.

Where a normal distribution could be assumed, mean value \bar{x} is stated and Student's *t*-test has been used. In other cases the median is stated and statistical analysis has been done by Mann-Whitney's *U*-test. N=patient material, P=control material, *p*=confidence limits, U=international units.

	Serum					Nasal secretion				
	Alb (g/l) \bar{x}	IgG (U/ml) \bar{x}	IgA (U/ml) \bar{x}	IgM (U/ml) \bar{x}	IgE (U/ml) median	Alb (g/l) \bar{x}	IgG (U/ml) median	IgA (U/ml) \bar{x}	IgM (U/ml) median	IgE (U/ml) median
N	42.0	121.8	100.5	126.8	0-10	3.4	2.7	12.3	—*	—
P	45.2	134.5	101.5	138.0	150	5.6	3.0	14.4	—*	—
Diff	3.2	12.7	1.0	11.2		2.2		2.1		
<i>p</i> <	0.003				0.01	0.004			0.10	0

* Median lower than measurement sensitivity of laboratory analysis.



Solitary IgE producing plasma cell. Immunofluorescence microscopy of biopsy from inferior turbinate in allergic rhinitis.

There were several fluorescent cells found (Fig. 1).

There was no correlation between the presence of plasma cells and the concentration of immunoglobulin fractions in the nasal secretion.

By investigating the relation between the presence of fluorescent plasma cells and immunoglobulin concentration in serum, both IgG and IgE fluorescent plasma cells were found in patients with considerably higher concentration of the comparable immunoglobulin (IgM: $0.02 > p > 0.01$, IgE: $p < 0.01$).

DISCUSSION

Qualitative identification of protein in nasal secretion has only been used a few times since Linn et al. (1972) found a method for this, based on weighing the secretion picked up on paper strips. In previous investigations immunoglobulin concentration was measured in relation to the concentration of albumin in nasal wash out fluid, which has been regarded as a rather constant factor. As shown by Lind et al. (1975) the concentration of albumin varies considerably, and in pathologi-

cal conditions in the nose, the concentration will increase, as the nasal mucosa becomes permeable to protein, a fact which we also found in our investigation.

This leakage through the mucosa is confirmed by the fact that some patients with high albumin concentrations (P 4, P 11 and P 23) had also a very high concentration of a number of immunoglobulins in their secretion. We are not able to explain why we found a higher serum-albumin concentration in the patient group than in the normal material, but the difference was statistically significant ($p < 0.003$).

The ratio of $IgG_{secretion}/IgG_{serum}$ and $IgA_{secretion}/IgA_{serum}$ was identical in both the patient group and controls, which indicates that no change in the local production of these globulins is found in perennial rhinitis. That the ratio $IgA_{secretion}/IgG_{secretion}$ is approximately 7 times larger than the ratio IgA_{serum}/IgG_{serum} is due partly to the fact that IgA is produced locally to a considerable degree, and is also secreted actively through the glands of the nasal mucosa, as two IgA molecules are linked with a "secretory piece" (Brandtzaeg, 1973). In the microscopic investigation of the mucosa we have found a number of plasma cells along the bottom of the gland acini.

One patient (P 21) had extremely low concentrations of IgA in both serum and nasal secretion. This was a 24-year old male patient who had no unusual history in comparison with the others. His nasal symptoms were moderate, and he had no history of immunological deficiency. All other examinations were negative.

The concentration of IgM in nasal secretions has previously been investigated on a few occasions. Under normal circumstances it is not excreted in nasal secretions (Linn et al., 1972), a fact which is shown in our control subjects. Probably due to the leakage mentioned above, we have in the patient group shown concentration of IgM in secretion, the difference being of low significance ($p < 0.01$). IgM producing plasma cells were found in 1/3 of the patients, and it is notable that these had



Fig. 4 IgE producing cells gathered in groups in fluorescent microscopy of inferior turbinate in perennial rhinitis

significantly higher serum concentrations of IgM than the others ($0.02 > p > 0.01$). This could be an indication of a generally increased activity within the IgM plasma cell population in the body. There was no proof that this increased IgM activity could be due to some sort of compensation for a poorly developed IgA system, as these patients did not differ from the others with regard to concentration of IgA in serum or secretion.

Concentration of IgE was found increased in serum ($p < 0.01$) and nasal secretion ($p < 0.1$) in the patient group. Corresponding changes were shown in perennial rhinitis by Handrock & Baumgartel (1975). IgE-producing plasma cells in larger amounts have not been shown in the patient group and the controls, but as was the case with IgM there was a tendency for a higher concentration of IgE in serum in patients where IgE fluorescent plasma cells were present than in the others ($p < 0.1$), which may indicate a general increase of IgE plasma cell population in perennial rhinitis.

Identification of allergen specific IgE antibodies (RAST) in serum is of limited interest in perennial rhinitis, probably due to the small

size of the target organ. One could hope that RAST investigation of nasal secretion could be of great value in finding in which patients the allergic mechanism is of importance (allergic rhinitis), which patients an unspecific, vasomotor instability is of importance (vasomotor rhinitis).

Huggins & Brostoff (1975) have investigated a group of patients with positive nasal secretion tests and specific antibodies in nasal secretion against house dust mite, negative prick test and negative RAST in serum. In our investigation by RAST of nasal secretions, we, however, obtained poorly reproducible and weak reactions which could not be interpreted satisfactorily. The same result has been reported by H. Levy (1975).

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We should like to extend our thanks to the Laboratory at Aalborg Sygehus Syd for very good cooperation in the laboratory investigations. The immunofluorescent microscopic investigations were done at the Institute at Aalborg Sygehus Nord. Pharmacia supplied us with kits and helped with the total specific IgE localization.

ZUSAMMENFASSUNG

aktive Bestimmung von Albumin, Immunglobulin

Plasmazellen bestimmen. Das Vorhandensein des IgA im Nasensekret und im Serum bot nichts an, da es bestand kein Unterschied zwischen der Normalgruppe und der Patientengruppe. Es ist wahrscheinlich wegen der vergrößerten Permeabilität der Schleimhaut im Nasensekret der Patienten nachweisbar. Diese Annahme stützt sich darauf, dass Albumin ebenfalls in wesentlich höherer Konzentration im Patientenmaterial vorgefunden wurde. Das Vorhandensein der IgM produzierenden Plasmazellen war je nach Patientengruppe als im Normalma und bei den Patienten, bei denen diese Zellen nachgewiesen wurden, fand man höhere Konzentration des IgM im Serum als bei den übrigen Patienten. Was auf eine allgemein gesteigerte IgM Aktivität deutet. Die Bestimmung des antigenspezifischen IgE (RAST) im Sekret ergab schwer reproduzierbare und unsichere Resultate, die auf die entsprechende Nachprüfung des Serums nicht zurückgeführt werden konnten.

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THE NEUROMUSCULAR PHONATORY CONTROL SYSTEM AND VOCAL FUNCTION

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Abstract This paper reports on recent studies supposed to answer the question whether there exists a relationship between the ability of kinesthetic voice control i.e. the operational efficiency of the neuromuscular phonatory control system and the quality of vocal function. In three subject groups with differing qualities of vocal function (24 singers 33 vocally untrained persons and 30 patients with hyperfunctional dysphonia) pitch and intensity changes of the speaking and singing voice were studied during interruption of auditory feedback by increasing binaural masking (white noise of 80 100 and 110 dB SPL) by means of our recently developed fundamental frequency analyser. The following results were obtained: the increase in speech intensity level was similar in all groups but the elevation of mean speaking fundamental frequency differed significantly: the dysphonic group reacted to binaural masking with the greatest pitch increments and the vocally trained group (singers) with the least ones. Additionally dysphonic patients showed significant reduction in pitch variations (speech melody) voice accuracy i.e. the ability to reproduce a given reference tone under masking conditions.

highest incidence of an incorrect pitch regulation ("initial overshoot") during sudden upward shifts of fundamental frequency. From these observations and on the basis of physiological data about pitch and intensity control the following conclusions are drawn: the neuromuscular phonatory control system works more efficiently in vocally trained persons than in vocally untrained ones or especially in dysphonic patients thus revealing that kinesthetic control ability is closely related to the quality of vocal function. As this ability can be measured by observing pitch changes under masking conditions an objective evaluation of the individual vocal function by means of fundamental frequency analysis seems feasible.

The production of a vocal sound requires properly coordinated movements of numerous muscles of the larynx, the vocal tract, the thoracic wall and the abdomen, the precision with which this coordination is achieved deter-

mines the quality of vocal function. In words, vocal function will be the better, the more precisely the activity of synergistic and antagonistic muscles is balanced during phonation. Any discoordination by excessive or insufficient tension or relaxation of synergists and antagonists or adjacent muscle groups will impede the accurate tuning of the phonatory apparatus, not only deteriorating voice quality but also increasing vocal strain which may become the basic reason for the development of a dysfunctional vocal order. As the phonatory process is controlled by a nervous control circuit (Fig. 1) the function depends entirely upon the operational efficiency of this system. It is well known from empirical evidence and experiments (Hanley & Steer, 1949, Atkinson 1951, Mann & Tegtmeyer, 1961, Morgan et al., 1969, Klingholz et al., 1973) that an interruption of the main control loop i.e. the auditory feedback, causes various disturbances upon vocal performance. However, as the automonitoring of vocal output is only a part of the phonatory control system, clearly shown by the fact that in spite of a partial or total loss of hearing an adult continues to phonate and articulate normally, although several voice and speech parameters like vocal pitch and intensity, speech melody or speech velocity (Petersen & Arnold 1970) become somewhat altered. Another control loop is represented by a neuromuscular reflex system being summarized under the term "kinesthetic"

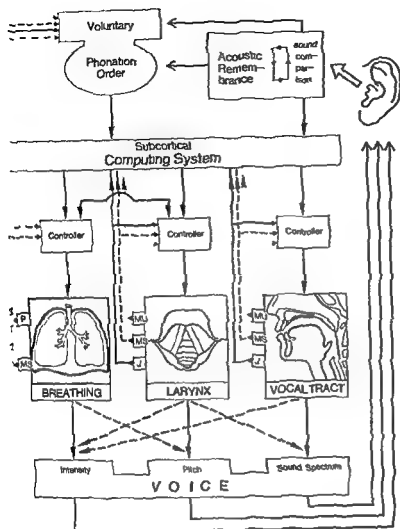


Fig 1 Schematic diagram of the phonatory control system *MU* mucosal mechanoreceptors *MS* = myotatic mechanoreceptors *J* = articular mechanoreceptors *P* pulmonary mechanoreceptors

" This system controls the motor action of the phonatory process almost automatically. Different types of mechanoreceptors in the vocal membrane, the muscles and the joints of the larynx, the thoracic and abdominal wall and of the vocal tract monitor muscular activity and movements during phonation. The total discharge from these receptors is fed back to the motoneurone pools in the brain, operating as individual controllers for laryngeal action and vocal tract movement as well as to an overriding subcortical computing system which controls the entire phonatory process according to the voluntarily given phonation order (Fig 1). All

though this neuromuscular control circuit works entirely below the conscious level, kinesthetic feedback also reaches cortical regions to a certain extent where it is consciously perceived as an overall idea of the present activity status of the phonatory apparatus (one can feel one's own voice).

The neuromuscular reflex system has long been regarded as of minor importance for phonatory control when compared with the dominant role of auditory feedback, but scientific work, especially that of Wyke and co-workers (Wyke, 1974a, b, Kirchner & Wyke, 1965, Kirchner & Suzuki, 1968) during the past decade strongly suggests that its opera-

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mines the quality of vocal function. In words, vocal function will be the more precisely the activity of synergistic and antagonistic muscles in balanced phonation. Any discoordination by excessive or insufficient tension or relaxation of synergists and antagonists or adjacent muscle groups will impede the accurate tuning of the phonatory apparatus, not only deteriorating voice quality but also increasing vocal fatigue which may become the basic reason for the development of a dysfunctional vocal order. As the phonatory process is controlled by a nervous control circuit (Fig. 1) the function depends entirely upon the operational efficiency of this system. It is well known from empirical evidence and experimental studies (Hanley & Steer 1949, Atkinson 1951, Mann & Tegtmeyer, 1961, Morgon et al. 1965, Hanley & Harvey, 1965, Charlip & 1969, Klingholz et al., 1973) that an interruption of the main control loop i.e. the auditory feedback, causes various disturbing effects upon vocal performance. However, the automatic monitoring of vocal output is only a part of the phonatory control system, clearly shown by the fact that in spite of a partial or complete hearing impairment or loss of hearing an individual continues to phonate and articulate normally, although several voice and speech parameters like vocal pitch and speech melody or speech velocity (Petersen & Arnold 1970) become somewhat altered. Another control loop is represented by a neuromuscular reflex system being summarized under the term "kinesthetic

Key words:

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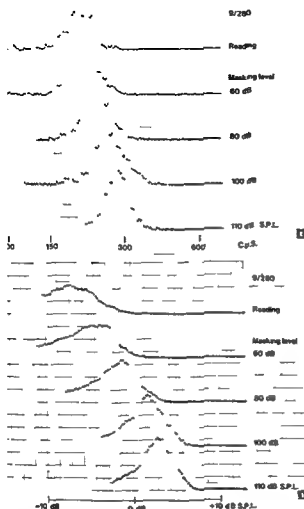


Fig 3 Increase in median fundamental frequency (A) and median intensity level (B) of the speaking voice due to increasing binaural masking (female subject). The median pitch and intensity levels are defined by the peaks of frequency and intensity histograms respectively. Note the changing shape of histograms indicating a reduction in pitch (and intensity) variation.

channel tape recorder (Revox A77) by use of a contact microphone (Fig 2). For measurement of the median pitch level the pitch range (i.e. difference between the highest and the lowest fundamental frequency of a given sample) and the median intensity level we used our fundamental frequency analyser (Fedders & Schultz Coulon) which measures the fundamental frequency and the sound pressure level of a voice continuously and simultaneously. The signal of this analyser was fed into a digital data processing system (DIDAC 800) which computed pitch and intensity distribution curves (histograms) for each sample. In addition we directly recorded pitch and inten-

sity curves of all singing voice samples on an UV light beam oscilloscope (Visicorder 2206) in order to obtain further information about tone stability, the character of pitch variations and the precision of fundamental frequency shift from one lower to the next higher tone.

2 Results

First we studied the changes of fundamental speech frequency and intensity under masking conditions. The example of an individual analysis is shown in Fig 3 (A, B) demonstrating that the individual reacts to the increase in masking noise level with rising elevation of both the median pitch and median intensity

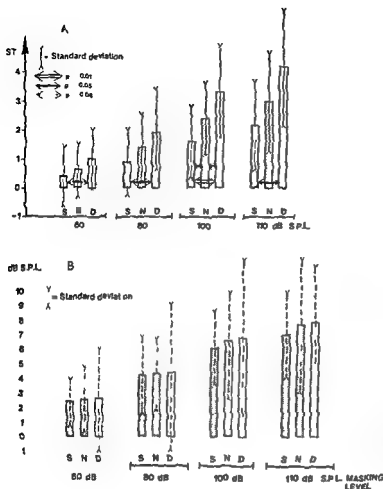
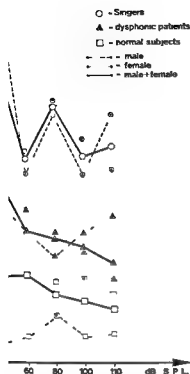


Fig 4 The average elevation of speaking fundamental frequency median intensity level (B) at different noise levels. S=singers, N=vocally untrained subjects, D=dysphonic patients. Statistically significant differences are indicated by arrows between

level (as defined by the peaks of the frequency and intensity histograms). This reaction known as Lombard Reflex (Lombard, 1911) was seen in all subjects tested for this study, but varied markedly with respect to the degree of pitch and intensity shift. Consequently, average data calculated for each subject group showed wide standard deviations, but they revealed the interesting finding that the mean values of pitch elevation differed considerably between groups, whereas those of intensity increase did not (Fig 4A, B). Singers elevated their fundamental speech frequency to a much lesser degree than the vocally untrained group, and the dysphonic patients showed the greatest frequency shift. When analysed statistically, the differences in pitch elevation between groups turned out to be significant, as indicated in Fig 4A (arrows between the columns in the diagram), whereas the slight

differences between the mean values of intensity increase were not statistically significant.

Speech melody, i.e. the variation of fundamental speech frequency, is said to become somewhat monotonous when auditory feedback is interrupted (Fris Winckel, 1957). In most cases we find the same observation (for instance, in Fig 4B) decrease in pitch modulation is demonstrated by the changing shape of the frequency histograms with increasing intensity of noise: they become taller, steeper and more slender, but in some cases we find opposite reactions or no change in modulation at all. As a quantitative expression of pitch modulation we took the ratio of base width to the height of a frequency histogram and called it "modulation quotient". Statistical analysis revealed that the speech melody was reduced in all three



ie average reduction in speaking pitch variation
reasing masking conditions MQ modulation
ie the ratio of the base width to the height
ency histogram the smaller the MQ the more
us the voice

groups (Fig 5), but that this reduction could be confirmed statistically (beyond the 5% level of significance) for the dysphonic group only

The accuracy of tone reproduction under masking conditions was measured by comparing median pitch levels of phonated tones (as documented by frequency histograms, see Fig 6) with the fundamental frequencies of reference tones (from an electronic organ), the results of these measurements being summarized in the diagram of Fig 7 with and without binaural masking the singers showed the smallest (mean 0.21–0.29 semitones) and the dysphonic group the greatest (mean 0.46–0.75 semitones) pitch deviations from reference tones, the difference between the normal subjects and the dysphonic patients not being a very distinct one, however. In singers, only a slight deterioration of voice accuracy can be noted with increased masking noise intensity, whereas in the vocally untrained and especially in the dysphonic group the median pitch deviation grows rapidly, reaching three-quarters of a semitone at a masking noise level of 110 dB SPL in both groups. Statistical analysis disclosed that this deterioration of voice accuracy under masking conditions

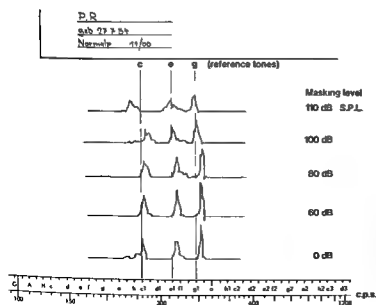


Fig 6 Individual analysis of the tone reproduction trial (female subject). Vertical lines indicate reference tones. Median pitch levels of phonated tones are defined by peaks in the frequency histograms.

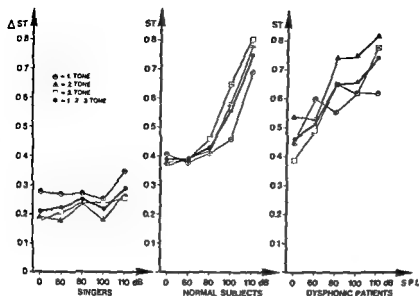


Fig 7 The dependence of accuracy upon masking ΔST = mean frequency deviation of reproduced tone from the reference tone ST = semitones

differed significantly between the singers and both the other groups (beyond the 5% level of significance), between normal and dysphonic persons no significant difference could be found, however

Regarding the influence of binaural masking on tone stability the singers can no longer be compared with the vocally untrained groups, all the singers sang with a regular modulation of fundamental frequency known as "vibrato", whereas all other persons showed the physiological irregular pitch fluctuations when sustaining a tone. Most singers kept the frequency as well as the regularity of their vibrato unchanged in spite of being masked,

only in a few cases did the vibrato bear somewhat irregular when the masking level reached 100 or 110 dB SPL. All groups showed a more or less pronounced "beating of the frequency modulation" ("Zitterbewegung"), which increased on average from 1.5 semitones (without masking) to 2.7 semitones (at a noise level of 110 dB SPL; see Fig 8). In both the other groups we observed a widening of the irregular frequency fluctuations upon increasing masking, but the most remarkable finding was the highly significant ($p < 0.01$) difference of tone stability between these two groups' frequency histograms.

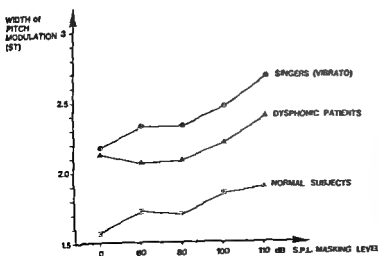
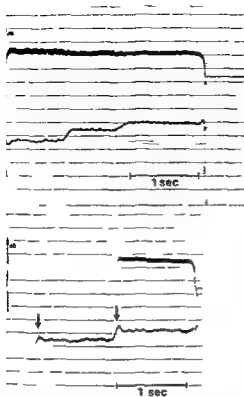


Fig 8 The widening of vibrato (singers) and of irregular pitch fluctuations (normal and dysphonic subjects) upon increasing masking conditions. Note the marked difference between normal and the dysphonic group ST = semitones



Fundamental frequency analysis of tone sequences of two different subjects. In (A) (female subject with noise level 100 dB SPL) pitch is accurately the lower to the next higher tone whereas the subject in (B) (masking noise level 110 dB) shows an initial overswing of fundamental frequency (Hz cps).

a much wider range of pitch fluctuation in dysphonic patients than in the normal. On average this difference amounted to approximately 5 semitones.

In addition, we would like to mention an observation concerning the precision of pitch control when the fundamental frequency is suddenly shifted from a lower to a higher tone. When reviewing pitch recordings of (singing) voice samples we noticed that any subjects that they did not change to the next reference tone but instead shifted vocal pitch upwards a little too far (sometimes by more than two semitones) thus having to lower it again in order to reach the actually wanted tone (Fig. 9B).

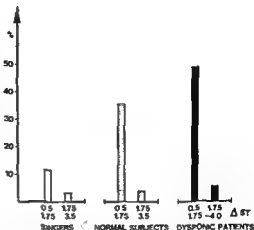


Fig. 10 The incidence of initial overswing of fundamental frequency in percent [ΔST =difference (sem tones) between the maximum pitch deviation of initial overswing and the median pitch of the entire tone].

The interesting point of this observation is the fact that the incidence of this phenomenon which we called 'initial overswing of fundamental frequency' was found to be highest in the dysphonic patients and lowest in singers without showing any dependence upon the masking situation. Fig. 10 compares the incidence of initial overswing among all pitch changes recorded (ten pitch changes per person). The diagram not only reveals that inaccurate pitch changes most often occurred in the dysphonic group but also that initial overswing tended to be more pronounced in this group than in the other two.

DISCUSSION

The vocal reaction in noise has to be regarded as a physiological reflex with the purpose of maintaining the possibility of verbal communication and at the same time, re-establishing acoustic automonitoring or—as Black (1950) put it—as the attempt of the human monitoring system to keep a normal experience at the speaker's ear. This purpose could be achieved by an increase in vocal intensity only, not necessarily requiring a simultaneous elevation of vocal pitch; however, for physiological reasons all people will more or less raise vocal

pitch when speaking with a louder voice. As it is seen from our study there is no proportionality between increase in vocal intensity and pitch elevation: some people raise their fundamental speech frequency in a noisy environment (or when being masked binaurally) much more than necessary, whereas others hardly change vocal pitch at all in spite of speaking markedly louder.

With regard to pitch and intensity control, we know from several aerodynamic and electrophysiological studies (Faaborg-Anderson, 1957, Rubin, 1963, Isshiki, 1964, 1969, Faaborg-Anderson et al 1967, Hirano et al 1969, 1970, Perkins & Yanagihara, 1968, Koyama et al 1971, Gay et al 1972 and other authors) that, at least at low and middle pitches, intensity is controlled predominantly by aerodynamic forces, i.e. by the activity of the expiratory muscle groups, whereas pitch depends mainly upon tension, length and mass of the vocal cords, i.e. upon the activity of

intrinsic and extrinsic laryngeal muscles. On the basis of this evidence, we have to conclude that raising the fundamental speech frequency needs a much higher activity of laryngeal muscles than intensity increments or that, in other words, speaking with a too high-pitched voice means considerably greater vocal strain than increasing vocal intensity. Consequently, those people who tend to excessive pitch elevation in noise must be particularly predisposed to the development of dysphonia. Briess (1957) suggested that "persons who raise their natural speaking voice will upset the dynamic equilibrium of the vocal muscles and develop voice difficulties". Brodnitz (1961) and Greene (1964) pointed out that heightened laryngeal muscle activity during phonation may be disruptive to optimal vocal function, and Dieroff & Siegert (1966) stressed the shift of fundamental speech frequency in a noisy environment as an etiological factor for the development of functional voice disorders. A similar conclusion was made by Stone & Sharf (1973) on the basis of experimental data demonstrating that the voluntary use of atyp-

ical vocal pitch induced significantly more phonetic voice changes than speaking at 2 intensity levels. From this point of view excessive pitch increments accompanying increase in vocal intensity has to be regarded as the expression of a faulty and uneconomical regulation of the phonatory process. Therefore, our finding that the dysphonic patients reacted to the interruption of auditory feedback with the greatest shift and singers with the smallest shift of fundamental speech frequency strongly suggests that vocally trained persons and especially persons with hyperfunctional dysphonia have a less efficient neuromuscular phonatory control system than have singers. Obviously, training over many years has taught singers always to limit muscular tension to the required minimum, thus enabling them without hearing their own voice, to keep the pitch level as much as possible while increasing vocal intensity.

A flattening of the melodic accent accompanying voice is apparently a frequent accompanying phenomenon of pitch and intensity elevation during binaural masking and is probably related to the increased tension of laryngeal muscles, because the group with the highest pitch increments, i.e. the dysphonic patients, also showed a more distinct tendency to reduce pitch variations than both the other groups. It would therefore seem reasonable to take an excessive reduction of pitch variations under masking conditions as further proof of an uneconomically operating neuromuscular control system.

Other evidence for the relationship between kinesthetic control ability and vocal function is given by the results of our tone reproduction trials: voice accuracy during masking deteriorated significantly more in the vocally untrained and the dysphonic persons than in singers, the dysphonic group showing the most rapid deterioration. Further on, the fact that even without masking the significantly smallest deviation from pitch of the reference tone was for

s, suggests that in persons with good function not only the kinesthetic but also auditory control mechanism of phonation more efficiently than in other persons. It should be mentioned that the pitch deviation of 0.21 semitones from a tones—as found in singers without a pretty well corresponds to the result of Kerppola & Walle (1925) and is a smaller as measured by Sakurai & Oka (1966), who in normal subjects reported pitch deviation of 2% (around 0.33

significant widening of vibrato swing regular pitch fluctuations regularly seen in the three groups has two causes: first, the control of pitch modulation depends upon intensity. Gemelli et al (1954), Sjöström & Winckel (1974) have shown that the vibrato swing becomes more pronounced at intensity levels, we recently demonstrated that the irregular pitch fluctuations in a similar way (Schultz Coulon 1977). All tested subjects increased the intensity of their singing voice as well as of their speaking voice when being masked binaurally. Secondly, a disturbance or interruption of kinesthetic automonitoring additionally influences tone stability, this has been pointed out by Deutsch & Clarkson (1959) and by Elliot & Heller (1970), and it is supported by our observations that in some singers the vibrato swing became irregular at maximum singing noise levels. However, in the individual case it is impossible to decide which of the two factors may be the more important for the impairment of tone stability. On the other hand, the fact that the increase in pitch fluctuations upon masking remains relatively small in all three groups suggests that tone stability depends to a greater extent upon the kinesthetic control mechanism than upon auditory feedback. In this regard we interpret the remarkable difference in tone stability between the dysphonic group and the normal subjects (the average frequency range of pitch of the dysphonic exceeded that of

the normal group by about 0.5 semitones in all masking situations) as further evidence of an especially poor kinesthetic control ability in the dysphonic group.

The phenomenon of "initial overswing" as described above can be explained in two ways: either the sudden upward shift in fundamental frequency is accompanied by a short increase in the glottic air flow, causing the pitch elevation, or else the laryngeal muscles are initially adjusted to a higher activity level than the pitch of the new tone would require. In any case, an initial overswing has to be regarded as incorrect pitch regulation which obviously mirrors the operational efficiency of the neuromuscular control system, because it occurs as frequently without as with interruption of auditory feedback (this is easily understood, since most of the time an initial overswing can not be perceived acoustically). In our experiment such inaccurate pitch regulation was seen more often in dysphonic patients than in the normal group, but rarely in singers. This result again indicates that the neuromuscular control system works more efficiently in vocally trained people than in other ones.

On the basis of these observations the following conclusions are drawn. The ability of kinesthetic voice control appears to be closely related to the quality of vocal function. The individual kinesthetic control ability can be evaluated by measuring the elevation of fundamental speech frequency and the accuracy of the singing voice during binaural masking as well as by observing the pitch fluctuations of a sustained tone and the precision of pitch control during a sudden upward shift in fundamental frequency. Therefore the examination of kinesthetic control ability by means of fundamental frequency analysis seems to offer a possibility for obtaining objective information about the quality of vocal function. For the voice therapist such a possibility could become of particular value not only for diagnostic purposes but also for an objective documentation of the therapeutic success of vocal training.

ZUSAMMENFASSUNG

Die Arbeit berichtet über Untersuchungen zur Frage ob eine Beziehung zwischen der kinästhetisch reflektorischen Phonationskontrolle und der Qualität der Stimmfunktion existiert. Bei 3 Personengruppen mit qualitativ unterschiedlicher Stimmfunktion (24 Sanger, 32 stimmlich untrainierte Personen und 30 Patienten mit hyperfunktioneller Dysphonie) wurden Tonhöhen und Lautstärke gemessen.

Die Lautstärke von 60, 80, 100 und 110 dB mit Hilfe unseres kürzlich entwickelten Grundtonanalysators gemessen. Das Experiment führte zu folgenden Ergebnissen: während das Anwachsen der Sprechintensität bei allen drei Gruppen ähnlich groß war, unterschieden sie sich hinsichtlich der Anhebung der mittleren Sprechstimmlage signifikant. Die dysphonischen Patienten reagierten auf binaurale Vertaubung mit der größten Tonhöhenanhebung.

Sprechstimme. Die Stimmgenauigkeit d.h. die Fähigkeit einen gegebenen Referenzton unter Vertaubungsbedingungen zu reproduzieren, war bei Sängern weitaus größer als bei Normalpersonen und Dysphonikern. Unabhängig von der Vertaubungssituation wiesen dysphonische Patienten eine deutlich geringere Stabilität der Singstimme auf als die stimmgesunden Normalpersonen und zeigten auch am häufigsten eine unkorrekte Tonhöhenregulation (ein sog. initiales Überspringen) beim schnellen Übergehen von einem niedrigeren zu einem höheren Ton. Vor dem Hintergrund der derzeitigen physiologischen Kenntnisse über Tonhöhen- und Lautstärkekontrolle werden diese Beobachtungen als Nachweis dafür aufgefaßt, daß die kinästhetisch reflektorische Phonationskontrolle bei trainierten Stimmen besser entwickelt ist als bei stimmlich untrainierten und bei dysphonischen Patienten, woraus folgt, daß eine enge Beziehung zwischen der Leistungsfähigkeit der kinästhetisch reflektorischen Stimmfunktion und der Qualität der Stimmfunktion besteht. Da diese Leistungsfähigkeit durch Beobachtung der Sing- und Sprechtonhöhenveränderungen unter Vertaubungsbedingungen gemessen werden kann, erscheint eine objektive Bewertung der individuellen Stimmfunktion mittels der Grundtonanalyse möglich.

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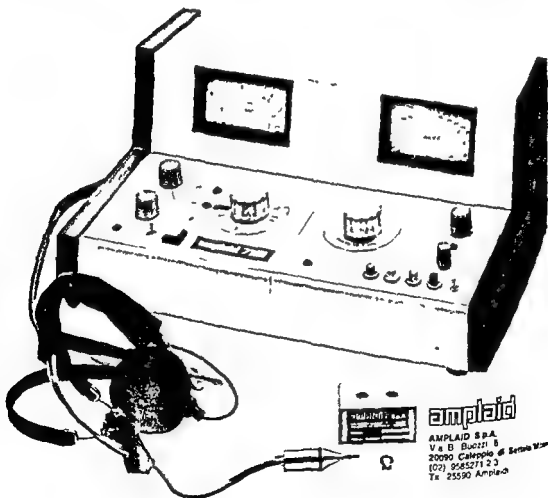
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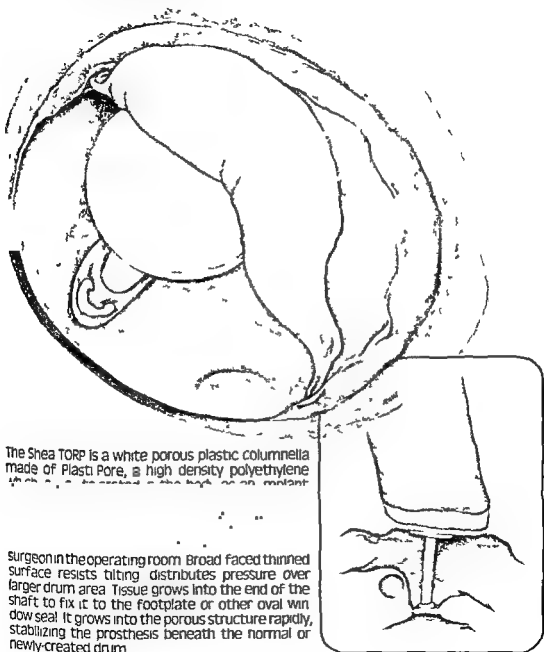


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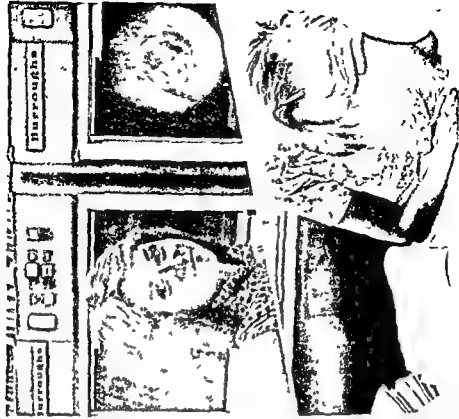
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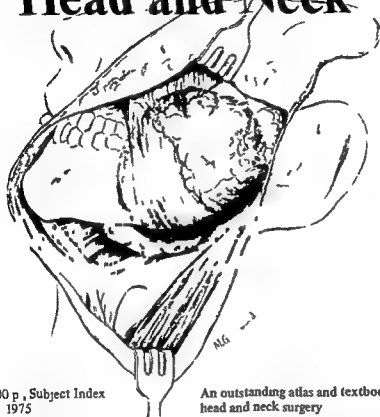
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PERIPHERAL VASOCONSTRICTION IN THE RAT IN RESPONSE TO SOUND

III *Dependence on Pause Characteristics in Continuous Noise*

E. Borg

From the Department of Physiology II Karolinska Institutet Stockholm Sweden

(Received September 9 1977)

tract The peripheral vascular reaction to sound was studied in the non anaesthetized rat. Arterial pulsations were recorded by a non invasive technique from the tail of the animal. The offset of a noise was found to be a stimulus for vasoconstriction eliciting responses in half of the presentations. A vasoconstriction was ever regularly elicited by onset of sound after the of the pause. The vasoconstriction was independent of pause duration in the range 10 ms to 100 s. For shorter pauses the reaction was smaller. The 2 ms pause elicited vasoconstriction only occasionally. It was especially noted that a 10 ms pause gave a larger response than a ms burst of noise. The results were discussed in relation to decay of sensation and partial masking effects.

sensitive to rapid changes than to slow changes in sound level (Borg 1978b).

The aim of the present study was to determine

(a) whether the acoustic vascular reflex is also sensitive to direction of change of sound level and

(b) whether it is dependent on the duration of a pause in a continuous noise in a way analogous to its dependence on the duration of a noise burst.

METHODS

Two series of experiments were performed whereby two different ranges of pause duration were investigated. 11 male adult rats participated in the first experiment and 10 in the second. Two animals were experimentally naive and 8 animals took part in both sessions.

Since a detailed presentation of the stimulus and recording system has been given earlier (Borg 1977) the following description of these will be brief. During the experimental session the animal rested on a regulated heating pad in an individually adjustable net tube. The bloodflow in the tail was assessed in terms of volume pulsations (Hellstrom 1975) recorded by a rubber balloon connected to a volume sensitive transducer (Elema 510C). In room temperature the tail artery was constricted and pulsations were minimal. A moderate heating was necessary to induce vasodilatation. The acoustic vascular reflex

of the rat has suitable characteristics for studies on the influence of sound on the cardiovascular system. With a non invasive technique arterial pulsations can be recorded in non anaesthetized animals (Borg 1977). A sound burst brings a drop in pulse amplitude whose duration rather than magnitude is used for a quantification of the response. Under these conditions the vascular response to a noise burst follows an approximately ear intensity function down to the threshold hearing (Borg 1977). The sound energy is integrated over time in such a way that noise bursts with durations of 10 ms give significant smaller responses than stimuli of 0.1 or 1 s duration (Borg 1978a). During prolonged stimulation the vasoconstriction slowly habituates after approximately 1 hour of exposure to 80 dB SPL noise; the pulse amplitude is completely normal (Borg 1978a). It has also been shown that the vasoconstriction is more

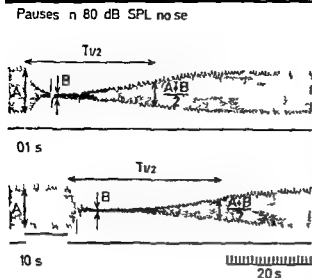


Fig. 1 Recordings of arterial pulsations obtained by a non invasive technique from the tail of a non anaesthetized rat. Decline of pulse amplitude selected by pauses in 80 dB SPL prolonged noise. Upper record 0.1 s pause. Lower record 10 s pause. The vasoconstriction is quantified by $T_{1/2}$ time from end of pause until pulse amplitude is halfway normalised.

manifested itself as a decrease of pulse amplitude usually to less than 10% of the prestimulus value. The vasoconstriction was quantified as the duration of the response from the start of the sound until the amplitude of the pulsation had returned halfway to prestimulus level ($T_{1/2}$ of Fig. 1). The reasons for using the duration of the response rather than the change in pulse amplitude as a measure of the vasoconstriction have been presented earlier (Borg 1977). A Lansing L75 loudspeaker placed 10 cm in front of the rat was used to deliver the sound stimuli.

The animals were exposed for about 1 h to a continuous broad band noise (spectrum see Borg 1977) at a level of 80 dB SPL (sound pressure level re 20 μ Pa). The stimuli consisted of pauses in the continuous noise (realized as 65 dB attenuation) of varying duration: 10 ms, 100 ms, 1 s, 10 s and 100 s in experiment 1; 2 ms, 5 ms, 10 ms and 100 ms in experiment 2. The rise and decay time of the noise breaks was 1 ms. In the first experiment the stimuli were usually presented only 1–2 times; in the second experiment they were pre-

sented 3–5 times with an interval of 2–4 min.

RESULTS

After approximately one hour's exposure to continuous broad band noise at 80 dB SPL the animal habituates with respect to peripheral vasoconstriction. If the noise is interrupted, however, the vessel constricts again. Fig. 1 shows two typical recordings of arterial pulsation obtained with the non invasive technique from the surface of the tail of a non anaesthetized rat. The animal had been exposed for several hours to continuous 80 dB SPL noise and the initial vasoconstriction had been habituated. Typical observations during 4 short 0.1 s and one long 10 s pause are illustrated. The gap in the ongoing noise is indicated in the lower trace.

It is seen that the pulse amplitude is low before the break, fluctuating only in respiration. The short pause elicits a constriction lasting for about 1 min. From recording it is not possible to decide whether the offset of the noise or the reiteration of noise after the interval is adequate stimulus for the vascular reaction. By using long pauses (10 s or 100 s) it is possible to

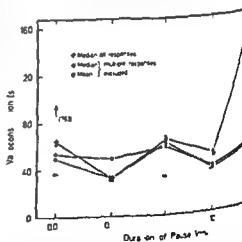


Fig. 2 Duration of vasoconstriction as a function of pause duration. Experiment 1: 10 ms to 100 ms. Median and semi interquartile range of all responses after multiple vasoconstrictions have been excluded. Measurements on 11 rats.



Fig. 2. Duration of vasoconstriction as a function of duration of pause in prolonged 80 dB SPL noise. Experiment 1: 2 ms to 100 ms pauses. Median $\pm 25\%$ (●) and \pm S.E.M. (○) of all responses. Measurements on 10

between the reactions to offset and onset of noise. In the lower recording in Fig. 1, it is seen to be elicited not by the offset of the noise but by its onset after the pause. However, in about half of the recordings there was in fact a reaction to halting the noise. This reaction was smaller, more variable and usually of shorter duration than the reaction to onset. At the end of the pause (offset of noise) a vasoconstriction was regularly observed. The offset of a sound is itself a weak stimulus for vasoconstriction in the rat under present conditions.

Fig. 1 the reactions to 0.1 s and 10 s pauses were nearly identical. The dependence of vasoconstriction on the duration of pause is more clearly illustrated in Fig. 2. This figure shows mean and median for the measurements in experiment 1: pause length 2 ms to 100 ms. The vasoconstriction was measured as illustrated in Fig. 1 by T_1 , the time from onset of noise until pulse had reached half way to prestimulus value. Since in 25% of the pauses were not followed by a usual monophasic vasoconstriction (see Fig. 1) but by several consecutive constrictions, two median values were calculated. One median value was based on all responses (+) and the other one did not include multiple responses (●). In addition, mean value (○) and standard error were calculated for the

latter sample. It is seen in Fig. 2 that pauses between 10 ms and 10 s do not differ systematically in their efficiency in causing vasoconstriction. If all the values are considered, a 100 s pause seems to give a longer response than the short pauses. This difference is not significant, however (in only 5 out of 11 animals did the 100 s stimuli give greater responses than did the 10 s stimuli). It is interesting to observe that the diminished efficiency in eliciting vasoconstriction so clearly seen for noise bursts with durations below 100 ms (Borg 1978a) is not seen for short pauses.

Thus, in the first experiment it was not possible to detect differences between short and long pauses. In the second experiment still shorter pauses were investigated in the range from 2 ms to 100 ms. Fig. 3 shows average and standard error together with median and semi-interquartile range for measurements in 10 rats. Only few responses were multiple and were not excluded. It is seen in Fig. 3 that vasoconstrictions were usually smaller for 5 ms than for 10 ms pauses; the difference is not significant ($p > 0.05$ Student's *t* test). The 2 ms pause (with a 1 ms rise and decay time) gave a significantly smaller vasoconstriction than the 10 ms pause ($p < 0.001$ Student's *t* test).

In conclusion, the offset of a continuous noise may elicit a vasoconstriction, but in most cases the vasoconstriction is caused by noise onset after the end of the pause. A pause as short as 10 ms gives responses of the same length as pauses of many seconds duration.

DISCUSSION

The present results show that the vasoconstriction reflex is differentially sensitive to onset and offset of noise, onset being a more efficient stimulus. On the other hand, the duration of the vasoconstriction is independent of the duration of the pause in the range 10 ms–100 s. Only for pauses shorter than 10 ms does vasoconstriction decline.

The present finding of the variability of

vasoconstriction on the offset of noise is in agreement with observations on heart rate response in rats made by Berg et al (1975). They found an acceleration of cardiac frequency on the halting of a tone, but only when a K-complex was elicited simultaneously in the electroencephalogram. Generally speaking, heart rate did not alter on halting the tone. Inglis (1974), on the other hand, found a significant average decrease in heart rate, both at onset and offset, of a 40 dB SPL noise. Only one presentation of the stimulus was made for each animal in this latter work. In the present study several pauses were presented and the animals were not experimentally naive. Habituation to offset may explain the discrepancy between the present results and those of Inglis (1974). There is, however, no clear evidence for habituation within the individual experiments in the present study. It seems rather to depend on the individual animal, some rats not showing responses to noise halt at all, and others doing so in a fairly consistent manner.

The most interesting finding of the present study is the very short integration time for reaction to a pause. If a pause is to be detected, it is necessary that the neural activity elicited by the noise decline rapidly at the beginning of the pause. In recordings from single fibres in the auditory nerve, it is evident that neural activity usually stops within a few ms after the end of a tone (Kiang, 1965). The spontaneous activity or the response to a subsequent test signal is, however, suppressed for 100 ms or more (Harns, 1977). The rate of decay of the auditory sensation has a similar time course. A temporal gap between two noise bursts is detected when it is longer than about 3 ms (Plomp, 1964) but decay of sensation is not complete until after about 200 ms. The present findings can be explained tentatively without assuming such differences between this reflex and perception.

In order to tentatively explain the present findings, two concepts of psycho-acoustics can be considered namely decay of sensation (see e.g. Plomp, 1964) and partial masking

(see e.g. Sharf, 1971). During the 200 ms takes for the gradual decay of sensation, the termination of a noise, an ensuing masked. It was shown (Plomp, 1964) that the sensation level of a 65 dB noise had decayed to 45 dB 10 ms after noise termination. If the same noise is presented again 10 ms, it will exceed the sensation level decaying sound by 20 dB. On the other hand, if a masked sound (Sharf, 1971) is 20 dB above its masked threshold, it has a loudness almost identical with the loudness of the same sound, unmasked. Even though these experiments are usually performed with a masking sound acting on another sound, it is difficult to see why the situation should be essentially the same for identical sounds. Consequently, one has to expect that the sensation level will already reach its maximum effect during the duration of 10 ms. Shorter pauses will be followed by smaller vasoconstrictions in the same way as the vasoconstriction is dependent on the level of the noise (Borg, 1977). Reservations must of course be made for differences between species and differences between vasoconstriction and perception, as well as for differences in the type of the stimuli used in the studies by Plomp (1964) and Sharf (1971) and in the present work.

If repeated vasoconstrictions in response to sound do cause permanent changes in the cardiovascular system, it can be supposed that the basis of the present experiments is that continuous steady noise would be less effective than an interrupted noise. Furthermore, steady noise with very short (e.g. 10 ms) pauses would give stronger vasoconstrictions than correspondingly short noise bursts (Borg, 1978a). The present findings tally with the assumption that the temporal pattern of noise is of greater importance than the level itself for bringing about cardiovascular

ZUSAMMENFASSUNG

Die Reaktion auf Geräuschstimuli in peripheren Gefäßen wurde an nichtanesthetisierten Ratten

der arteriellen Pulsationen im Schwanz mittels nicht invasiven Methode untersucht. Es zeigte sich ein plötzlicher Abbruch des Geräusches (Fall ms) mit Hinblick auf die Gefäßkontraktion als ein Reiz auswirkt, der nur in der Hälfte der Fälle auslöst. Durch Pausen in der Geräusch-
 1 wurden Gefäßkontraktionen ausgelöst, die ausenlangen von 10 ms bis 100 s unabhängig von der der Pausen waren. Für noch kürzere Pausen nahm ton ab und für 2 ms Pausen wurde nur gelegent ne Gefäßkontraktion ausgelöst. Besonders sei kt, daß eine Pause von 10 ms eine größere Reaktion als ein Geräuschstoß von 10 ms

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THE ULTRASTRUCTURE OF THE BASILAR MEMBRANE IN THE CAT

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Abstract A detailed study of the feline basilar membrane was performed in 13 cochleae with light microscopy and in six with electron microscopy. The distribution of the mesothelial cells and homogeneous ground substance with the filaments was recorded and plotted as a function of length along the cochlear duct. The width, thickness and number of filaments were also measured. In the lower basal turn the basilar membrane was narrowest and its entire thickness was occupied by filaments. In the apical region the width was maximal and the filaments were fewer. The density of the filaments counted in the bundles showed no significant difference along the cochlear duct or across the width of the basilar membrane, but the number of filaments decreased markedly (approximately a ten fold difference) from base to apex. The number of mesothelial cells increased towards the apex. These morphological characteristics may be related to the different motion pattern of the basilar membrane along the length of the cochlear duct. A discontinuity of the basilar membrane was noted in the apical region in all 19 cochleae studied. These gaps seemed to provide structural evidence for the permeability of the basilar membrane in this area. The vas spirale was present as a blood vessel in two specimens and only in the apical region. Thus its function as the sole nutritional source for the organ of Corti is doubtful.

The importance of the basilar membrane in the frequency selectivity of the cochlea has been recognized for more than a century (Helmholtz 1863, Hurst 1895, Zwislock 1953, von Békésy, 1960, Tonndorf 1960, Dallos 1970, Rhode, 1971, Wilson & Johnstone 1975). It is clear that different sound stimuli produce a maximum deflection of the basilar membrane at different levels of the cochlea and that the motion pattern of the basilar membrane is determined by its morphological features. These factors suggest that there are some structural differences in the basilar membrane along the length of the cochlear duct.

Other previous studies of the basilar membrane morphology (Retzius 1884 Engel 1955, Uvåga 1962, 1967, Aneelborg & Ekstrom 1974, Shinozaki & Miyoshi 1975) have been reported. In this study a detailed description of the different elements that comprise the basilar membrane is presented with emphasis on their distribution across the organ of Corti and along the length of the cochlea. The width, thickness and number of filaments were measured. The cat was chosen as the experimental animal since most of the available physiological and anatomical data are from this animal.

MATERIALS AND METHODS

A total of 19 cat cochleae were studied by light microscopy and 6 by electron microscopy.

Eight temporal bones from 4 adult weighing 2 to 3.5 kg were fixed in Hender Susa, embedded in celloidin (Sch 1968), and serially sectioned in the horizontal plane at 20 μ m thickness. Every tenth section was examined under the light microscope. The cochleae were reconstructed (Sch 1953). The average length of the basilar membrane was found to be $23.8 \text{ mm} \pm 0.96$.

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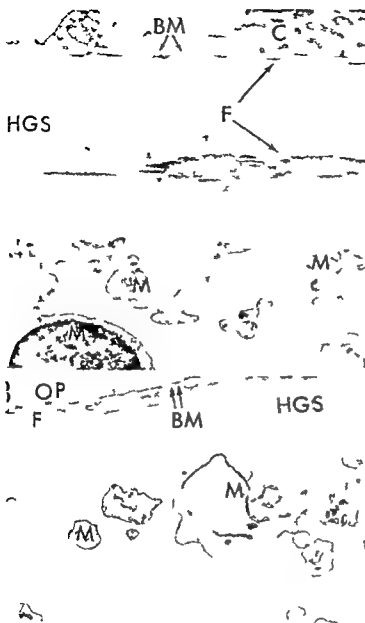


Fig 1 Electron micrographs showing the different elements that comprise the basilar membrane *BM* basement membrane *F* filaments (upper and lower layers) *M* mesothelial cells (A) Pars pectinata at the level of the Claudius cells (*C*) in the apical region $\times 8000$ (B) Pars tecta at the level of the outer pillar cell (*OP*) in the second turn. Note the different distribution of the filaments (*F*) they form a single layer at this level $\times 6750$

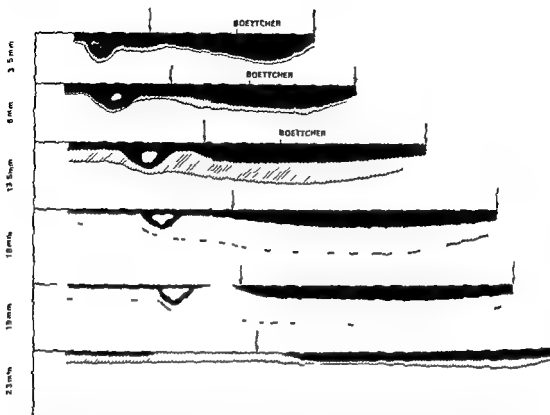
One ear from each of 5 adult cats was embedded in Epon and studied by surface preparation.

The width of the basilar membrane was determined from the nucleus of the inner pillar cell to the basilar crest (junction of the basilar membrane and spiral ligament). To the exact limits radial $2 \mu\text{m}$ thick sections were also made.

One ear from each of 6 cats was used for microscopic study. To allow direct

comparison between young and adult the animals were divided into two groups: one a group of 3 small animals (1.09 to 1.27 kg) and the second a group of 3 adult animals (3.09 to 3.4 kg).

The cochleae were intravitaly perfused through the opened oval and round windows with Karnovsky's solution (1965) at pH 7.3. The cochleae were postfixed in 1% phosphate buffered osmium tetroxide and embedded in



2 Schematic drawing showing the variation in thickness of the basilar membrane along the cochlear duct at different points in the same ear. The black and shaded regions represent the outline of the homogeneous ground substance with the filaments included and the area occu-

ped by the mesothelial cells respectively. The axis corresponds to the distance from the base. In each case the arrow on the right side indicates the crest and the left arrow indicates the demarcation between the pars tecta and the pars pectinata.

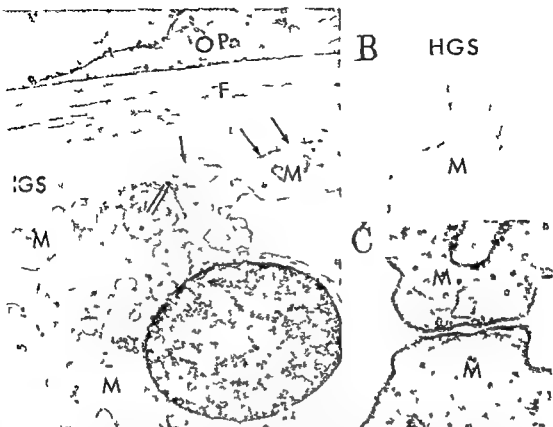
Epon (Kimura, 1967). Each embedded cochlea was then bisected in a plane perpendicular to the 5 mm point of a ruler placed against the round window and promontory. In all cases, two pieces were obtained from each turn corresponding to the following locations: lower basal turn, upper basal turn, lower second turn, and two from the apex. The location (± 1 mm) of each piece along the length of the cochlear duct was determined and plotted on a standardized cochleagram. In all specimens radial sections were made in a plane parallel to the long axis of the outer hair cells of the organ of Corti using an LKB Ultratome.

In two cochleae one additional piece was taken from each turn and was serially sectioned in a transverse plane to the basilar membrane, from the basilar crest of the spiral ligament to the habenula perforata. The sec-

tions were stained with uranyl acetate/citrate. Photographs were taken with a Siemens Elmiskop I at magnifications from 1000 to 40000 \times .

Measurements of the thickness of the membrane were made from the basilar membrane to the tympanic surface of the homogeneous ground substance and from points within the mesothelial cells at which seemed to best define the general shape of this area. Along the width of the membrane, an average of ten different points for each section was used to make the construction.

The density of filaments in the basement membrane ($F/\mu m^2$) was determined. The average number of filaments per micron length (F_{μ}) was obtained by counting the total number of filaments and relating that number to the



(A) Radial section of the basilar membrane at the level of the outer pillar cell (OPa). Occasionally hemidesmosomes (single arrows) are seen at the sites of contact between mesothelial cells (M) and the homogeneous ground substance (HGS). The double arrows indicate the junction between two mesothelial cells. (B) Higher magnification of the homogeneous ground substance (HGS). (C) Electron micrograph showing a desmosome-like junction between two mesothelial cells (M). Note that the intercellular space is smaller at the site of the junction.

×11200 (B) Higher magnification of the homogeneous ground substance (HGS) and the mesothelial cell (M) ×66000 (C) Electron micrograph showing a desmosome-like junction between two mesothelial cells (M). Note that the intercellular space is smaller at the site of the junction ×77000

basilar membrane contained within it. The distribution of filaments was defined by using a computerized polar planimeter.

FINDINGS

Ultrastructure of the Basilar Membrane

The electron micrographs of the basilar membrane underlines the organ of Corti from the tympanic lip of the limbus spiralis to the basilar crest of the spiral ligament. The mesothelial membrane forms a line of demarcation between the cells of the organ of Corti and the basilar membrane. Three main components can be distinguished: a fibrous portion, a homogeneous ground substance, and

the mesothelial cells (Fig. 1). The basilar membrane is divided into two zones: the pars tecta, which extends from the tympanic lip of the limbus spiralis to the outer pillar cell, and the pars pectinata, which extends from the outer pillar cell to the basilar crest of the spiral ligament.

The homogeneous ground substance has an amorphous and filamentous appearance. It lies between the basement membrane and the mesothelial cells. The fibrous portion is located within it. The distribution of the homogeneous ground substance (Fig. 2) varied not only along the length of the cochlear duct but also across the width of the basilar membrane.

Table II

	Filaments per μm^2			Filaments per μm length		
	Apex (23 mm)	Second (17 mm)	Basal (9 mm)	Apex (23 mm)	Second (17 mm)	Basal (9 mm)
Claudius						
Upper	1 580	1 664	1 400	137	697	777
Lower	1 440	1 460	1 450	343	655	958
Hensen						
Upper	1 358	1 479	1 504	112	527	656
Lower	1 945	1 548	1 400	364	876	935
Deiters						
Upper	1 622	1 231	1 592	74	453	607
Lower	1 441	1 359	1 375	402	949	1 089
Outer pillar	1 397	1 587	1 720	487	1 579	1 840
Tunnel	1 380	1 764	1 552	512	1 131	1 739
Inner pillar	900	1 200	1 269			

length of the cochlear duct (Fig. 2). There was an increase from base to apex and a decrease at the apical end of the cochlea. The maximum thickness was found in the upper second or in the lower apical turns. In the lower basal turn there was only one very thin layer (less than 1 μm thick) of cell nuclei and prolongations of each other and lying closely adjacent to the homogeneous ground substance. This appearance changed toward the apex and in the upper second turn three or more nuclear layers were present. Also the number of cell processes increased greatly with many free spaces between them.

The distribution of mesothelial cells across the width of the basilar membrane was very similar in both the pars tecta and the pars pectinata and it followed the pattern described above. The only differences were that in the pars tecta there were usually fewer cells and the area occupied by them was thinner than in the pars pectinata.

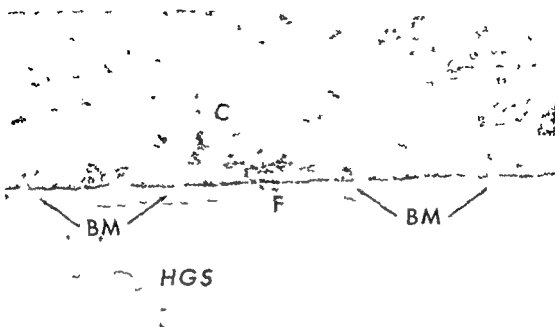
The total number of cells located across the width of the basilar membrane and the mean fraction of cells per micron width were determined by counting the nuclei in each radial section. There was an increase of mesothelial cells from base to apex except at the apical end of the cochlea where they decreased again. These values contrast with those found

by Angelborg & Engstrom (1974) in the guinea pig where the number of cells was always greater in the basal turn.

The fibrous portion was composed of radially oriented filaments with an uneven profile (Fig. 4B). The diameter of these filaments ranged from 90 to 120 \AA . Each filament was rectangular or diamond shaped in cross section with four or five subfibrils (Fig. 4C). Other investigators have found similar morphological characteristics in the vestibular perilymphatic space of rats (Horton 1967), the spiral ligament of the Rhesus monkey (Takahashi & Kimura 1970) and the basilar membrane of the guinea pig (Kamada 1975).

The distribution of filaments along the width of the basilar membrane differed in the pars tecta and the pars pectinata. In the pars tecta they lay side by side within the homogeneous ground substance forming a continuous compact layer while in the pars pectinata they were grouped in bundles and were separated by two or more strata. Homogeneous ground substance was present between the bundles in the two strata.

The density of the filaments in the two



6 Electron micrograph of the basilar membrane at level of the Claudius cells (C) in the apical region; arrows show the location of the basement membrane

(BM) HGS homogeneous ground substance F filaments $\times 19000$

the number of filaments per micron length in two cats are shown in Tables I and II. The density of filaments ($F/\mu m^2$) in the second turn showed no significant difference along the length of the cochlear duct (basal second turn) or across the width of the basilar membrane (1430 ± 250). However there was a decrease in density at the level of the outer pillar cell mainly in the apical region and in the second turn. In the tunnel of Corti some filaments appeared to change their radial orientation and assumed an oblique orientation. Therefore underneath the inner pillar cell the filaments were scattered and they appeared to be embedded in dense conglomerates of homogeneous ground substance.

The number of filaments were considered in relation to the length of the basilar membrane. No significant differences were observed not only across the width of the membrane but also along the cochlear duct. Across the width of the basilar membrane these dif-

ferences were more evident in the pars pectinata. In the upper layer the filament population decreased progressively from the basilar crest to the outer pillar cell while in the lower layer the opposite occurred (Tables I and II). This observation was in accord with the fact that the bundles in the upper layer became thinner. A discontinuity in the upper bundles was present (in both second and apical turns) at the level of the Hensen cell area. The lower layer on the other hand contained larger bundles. These complementary changes in the two strata arose from the presence of intermediate bundles which seemed to descend from the upper layer to the lower layer. In the lower basal turn it was not possible to obtain separate data for each strata because there were so many intermediate fibers that no discrete layers could be distinguished.

At the level of the Deiters cells both layers were very close and they joined underneath the outer pillar cell to form the pars tecta. At

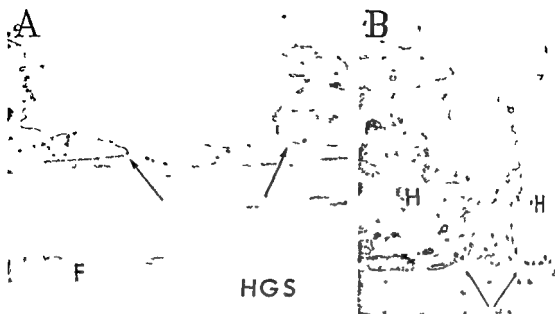


Fig 7 Electron micrographs showing the discontinuity of the basement membrane (arrows) at the level of the Hensen cells (A) Radial section in the 21 mm area (B) Cross section in the 23 mm area $\times 13700$

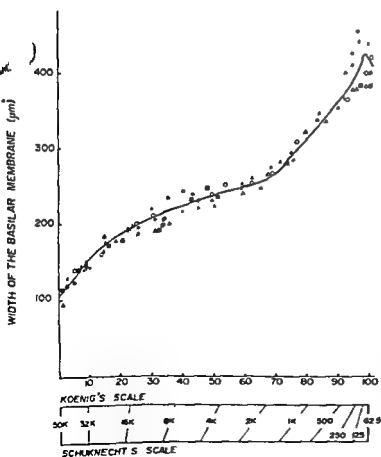


Fig 8 Graph showing the average width of the feline basilar membrane. The width in µm is plotted as a function of percentage of the cochlear length. The Koenig and Schuknecht frequency scales are presented along the bottom

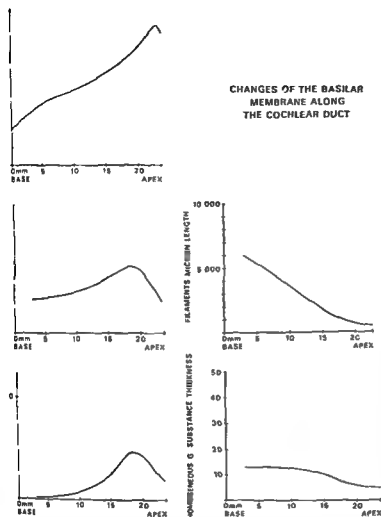


Fig 9 Composite diagram showing the changes of the basilar membrane and the elements that comprise it along the length of the cochlear duct. The graphs showing mesothelial cell homogeneous ground substance and total thicknesses were obtained at the level of the pars pectinata.

level of Corti's tunnel a group of filaments shed from this filamentous compact layer formed an arc around the *vas spiralis* area, which they again joined the filaments of the tecta. The number of filaments forming this arc was variable, that is they were numerous in the basal turn and very few in the apical region. The filaments per micron length were not determined at the level of the inner pillar cell because they were so dispersed that it was impossible to count the area containing filaments. The number of filaments per micron length varied greatly along the cochlear duct. In the lower basal turn (4 mm area) the entire thickness of the basilar membrane contained

filaments (5400 ± 700). The number decreased toward the apex, 1340 ± 70 in the upper second turn and 570 ± 110 in the apical end. It represents approximately a ten fold difference between base and apex. Therefore, the thickness of the area containing filaments decreased markedly along the length of the cochlear duct (Fig 5).

The *basement membrane* is a semi opaque linear structure of finely granular appearance which forms a boundary between the organ of Corti and the basilar membrane (Fig 6). It was separated from the supporting cells of the organ of Corti by a gap that varied between 200 and 400 Å. This space was randomly occupied by a granular material which seemed to have a

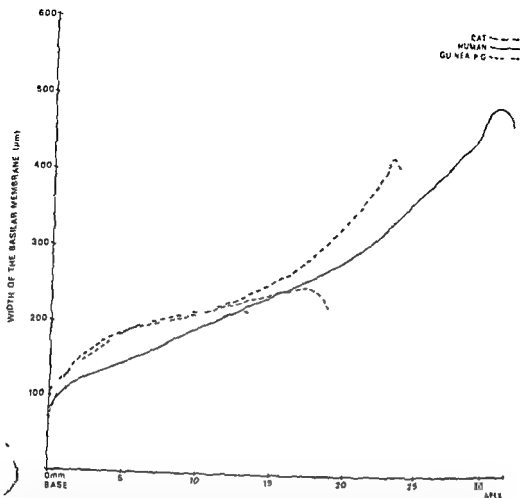


Fig. 10 Comparative graph of the variation in basilar membrane width for three different species. Human data were taken from Wever (1938, 1949) and guinea pig data from Fernandez (1952). The vertical axis corresponds to the width of the basilar membrane in μm and the horizontal axis to the length of the cochlear duct in mm: the human 18 mm for the guinea pig and 21.6 mm for the cat.

density comparable to the basement membrane itself. The thickness of the basement membrane ranged from 400 to 600 Å. No differences were found between the pars tecta and the pars pectinata.

In the upper second turn and in the apical region the basement membrane was discontinuous (Fig. 7). It folded over two adjacent Hensen cells and surrounded them, ending at the cell membrane and leaving a communication between the intercellular spaces of the organ of Corti and the basilar membrane. Also at this level the upper layer of filaments was very thin and a discontinuity between the bundles was observed. Such gaps were present after the 18 mm area in all the cochleae studied, and

in two cases they were even observed in the lower second turn. They varied greatly with a range of 0.09 to 1.8 μm . They combined with the loose distribution of mesothelial cells, would allow free communication between the perilymph with the intercellular spaces of the organ of Corti.

II The Width of the Basilar Membrane

Measurements of the width of the basilar membrane are shown in Fig. 8. The progressive increase from base to apex was noted in all cases. The maximum width was noted in the apical region. Other than individual variations in size of the curves, there were no significant differences that could be related to the age of the animal.

e curve representing the average width of basilar membrane showed a gradual increase from the round window to the lower end turn (65% of the cochlear length). At this level a sharp change in the slope of the curve was observed. This change occurred in the area between 1 and 2 kHz in the Koenig (1973) and Schuknecht (1974) scales. This sharp increase in the width of the basilar membrane was maintained until the maximum was reached. A sudden decrease in width was observed in the last 1 mm.

The maximum mean width of the basilar membrane was 426 μm and the minimum was 106 μm , which represents an approximately four-fold increase from base to apex.

II Thickness of the Basilar Membrane

The total thickness of the basilar membrane depended on the distribution of the homogeneous ground substance with the filaments of the mesothelial cells (Fig. 2). It should be noted that they varied conversely along the cochlea, thus, as the thickness of the homogeneous ground substance and filaments decreased from base to apex, the area corresponding to the mesothelial cells increased. The total thickness of the basilar membrane showed a progressive increase from the basal turn to the apical region, essentially due to an increase in the population of mesothelial cells. The maximum thickness was found at the level of the upper second or in the lower apical turn. At the apical end of the cochlea the total thickness decreased again.

DISCUSSION

The importance of the basilar membrane in the mechanical behavior of the cochlear partition has been recognized for more than a century. Imholtz (1863) formulated the resonance theory, suggesting that every tone has a specific locus along the basilar membrane where the fibers would act as resonators. Hurst (1938), on the other hand, suggested the traveling-wave theory and considered that the

wave of displacement progressed systematically along the basilar membrane producing a local stimulation in its path. Von Békésy (1960) made direct measurements of the vibratory characteristics of the cochlear partition. He showed that the sound stimulus produced a traveling wave and that each frequency maximally excited a different part of the cochlea. Furthermore, the maximum deflection of the cochlear partition produced by high frequencies occurred in the basal portion, whereas that produced by low frequencies occurred in the apical region. Wever et al. (1954) and Tonndorf (1959) established that traveling waves were produced by the transfer of energy from one perilymphatic scala to the other across the partition. However, the vibratory pattern was determined by the morphological properties of the membranous partition itself and would also be determined by the properties of the basilar membrane. Therefore, if the mechanical behavior of the cochlear partition varied from base to apex, this might be correlated with some morphological changes in the basilar membrane along the cochlear duct (Fig. 9). Such changes would determine the relative stiffness or elasticity of the basilar membrane at different levels.

After von Békésy's experiments, it was known that the basilar membrane is not under lateral tension, but it has some stiffness or resistance to displacement, which is one hundred times greater in the base than in the apex in the human (von Békésy, 1960). This difference can be explained, at least partially, by the differences in width, thickness and number of filaments along the length of the cochlear duct. Many previous studies have been concerned with the width of the basilar membrane (Hensen, 1863; Guild, 1927; Wever, 1938; Perlman, 1946; Fernandez, 1952; Igarashi et al., 1968; Antoli-Candela et al., 1976). A progressive increase from base to apex was always reported. In this study the width of the basilar membrane was also measured and the results were similar (Fig. 8). The narrowest width (106 μm) was located in the

basal region and the maximum (406 μm) in the apex (approximately a four fold difference). The increase in width of the basilar membrane was not gradually progressive. A sharp change in the slope of the curve was observed at about 15 mm from the basal end of the cochlea.

Although lateral tension has not been demonstrated in the basilar membrane, it is probable that an important contribution to the resistance to displacement of the membrane could be provided by the radial filaments. The density of the filaments within the bundles remained the same along the length of the cochlear duct, but the number of filaments which occupied the thickness of the basilar membrane underwent a great reduction from base to apex. At the lower basal turn, they numbered roughly 6000/ μm which contrasted with the 600/ μm in the apical region (approximately a ten fold difference).

The thickness of the basilar membrane (Fig. 2) increased from base to apex with a maximum at the level of the upper second turn or the lower apical turn. This change in thickness is correlated with the increased number of mesothelial cells towards the apex. Unfortunately, the role of the mesothelial cells in relation to the stiffness of the basilar membrane is not known. A further point of interest is the loose distribution of the mesothelial cells in the apical region. At this level they comprised the main element in terms of thickness. It is possible that in this apparently weaker portion of the basilar membrane where the homogeneous ground substance was very thin or absent in some areas and the filaments were fewer, they provide some kind of flexible support that acts as a cushion or energy absorber for strong vibrations.

Curves showing the mean widths of the basilar membrane for the cat, guinea pig and human appear in Fig. 10. The human data were obtained from Wever (1938, 1949) and the guinea pig data from Fernandez (1952). The shapes of these curves are similar. Each curve shows an increase in width from base to apex, however, each has its own characteristic rate

of interest. The minimum values for the species are more similar than the maximum values. At the basal end the values are 80 μm for the human, 106 μm for the cat, and 70 μm for the guinea pig. At about a half turn from the basal end where the maximum width is reached, the values are 498 μm for the human, 475 μm for the cat, and 250 μm for the guinea pig. When plotted against cochlear length the corresponding mean values are 32 mm distributed in 3 turns for the human, 23.8 mm in 3½ turns for the cat, and 18.8 mm in 4 turns for the guinea pig. The maximum width is noted in the apical region (Fig. 10) where the maximum vibration of the basilar membrane comes at the low frequencies. The limit for the mechanical frequency analysis at this level is 200 cps for the human and 200 cps in the guinea pig (Békésy, 1960). In the cat this limit is considered to be 62.5 cps using behavior (Schuknecht, 1953). It should be noted that these limits of the auditory field (for the human and guinea pig) are defined in terms of mechanical frequency analysis and further values obtained through neural processes.

Fleischer (1976) suggested that the maximum amplitude of displacement for mechanical frequency occurs at the same width of the basilar membrane in all mammals. This is improbable since the maximum width of the basilar membrane for the guinea pig is 250 μm which corresponds to the 14 mm area of the basilar membrane. At this area the frequency is about 1500 cps on the SD scale. If Fleischer's theory were correct it would imply that the guinea pig has a mechanical response to frequencies below 200 cps, and yet the accepted lower frequency limit is 200 cps. Therefore, other characteristics of the basilar membrane such as the number of filaments must also have an influence on mechanical frequency analysis.

Very few studies on the thickness of the basilar membrane in different species have been carried out. Fernandez (1952) found that the thickness of the homogeneous ground substance in the guinea pig was 7.4 μm .

ning of the first turn and $1.34 \mu\text{m}$ at the apical region but he did not mention at which place across the width of the basilar membrane measurements were obtained. In the present study an average of ten points for every section was used to make a graphic reconstruction. The maximum value for the homogeneous ground substance in the pars pectinata in the lower basal turn was roughly $14 \mu\text{m}$ and this value decreased to $5 \mu\text{m}$ in the apical region. If the total thickness were considered, the corresponding values would be 14 to $15 \mu\text{m}$ and $26 \mu\text{m}$ (upper second turn). Unfortunately, no data related to human basilar membrane thickness could be found. In all specimens studied, a discontinuity in the basement membrane between the Hensen cells was found in the apical region (usually around the 18 mm area). The possibility of a tectonic artifact due to drilling of the apex is overruled by the finding of these gaps in the cochlea in which there was no drilling. These gaps provide structural evidence of the communication between the intercellular spaces in the organ of Corti and the scala tympani through the basilar membrane, as previously described by Altmann & Walner (1950), Lindorf et al (1962), Nomura (1968), von Ilberg & Vosteen (1969), Masuda et al (1971), Lorenzo et al (1972) and Duvall & Sutherland (1972). At this level in the pars pectinata the upper layer of filaments was very thin and the discontinuity between the bundles was observed. The loose distribution of the mesothelial cells would allow free contact between the homogeneous ground substance and perilymph. Thus, with no barrier in the basilar membrane, such gaps could permit free passage of perilymph between the intercellular spaces of the organ of Corti and the scala tympani.

The vas spiralis, located beneath the tunnel of Corti, was first described by Retzius (1883) in the rabbit embryo and in the fetal cat. Smith (1954) performed a vascular study in 8 cats, and in 6 of them the vas spiralis did not present a blood vessel. It was therefore concluded

that the vas spiralis had little, if any, significance in itself. Lawrence (1966) reported that the interruption of blood flow through the vas spiralis in the guinea pig caused a loss of hair cells and suggested that it was an important source of nutrition for the organ of Corti. Kikuchi & Hilding (1967) considered that the vas spiralis in Shaker-1 mice underwent involution and lost its lumen 2 weeks after birth, which could be an important factor in the subsequent degeneration of the outer hair cells.

In this study the vas spiralis was observed in only two of the six specimens studied, and in both it was located in the apical region. Relating this observation with Retzius' findings (1884) which showed that this vessel disappeared in the apex in the 30-day fetal cat, though it was present in all coils at an earlier stage, it seems clear that the vas spiralis undergoes involution during the cat fetal period. Thus, when the vas spiralis remains in the adult animal, it could probably be considered an embryo-vestige. No correlation with the age of the animals (young or adult) was noted, nor was there any obvious pathology at the level of the organ of Corti. Therefore, the function of the vas spiralis as the sole nutritional source for the organ of Corti is doubtful.

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ZUSAMMENFASSUNG

In 6 elektronenmikroskopischen und 13 lichtmikroskopischen Cochlea Präparaten wurde eine eingehende histologische Untersuchung der Membrana basilar vorgenommen. Die Verteilung von Mesotheliumzellen und der von Filamenten durchsetzten Grundsubstanz wurde als Funktion zur Längendimension des Ductus cochlearis dargestellt. Die Weite, Dicke und Zahl der Filamente wurde ausgemessen. In der untern Basalwindung, wo die Basalmembran am schmalsten ist, ist sie völlig mit Filamenten durchsetzt. In der Spitzenwindung, wo die Basi-

larmembran am breitesten ist sind die Filamente weniger zahlreich. In der Bündeldichte der Filamente wurden keine signifikanten Unterschiede langs des Ductus cochlearis festgestellt jedoch nahm die Zahl der Filamente von der Basalwindung gegen die Spitzenwindung etwa um das Zehnfache ab. Andererseits nahm die Zahl der Mesotheliumzellen gegen die Spitzenwindung hin zu. Diese morphologischen Unterschiede korrelieren wahrscheinlich funktionell mit einem differentiellen Bewegungsmuster der Basalmembran. In allen untersuchten Spezimen wurden in der Apikalwindung Diskontinuitäten in der Basalmembran gefunden. Diese Lucken weisen auf spezifische Permeabilitätseigenschaften der Basalmembran in der apikalen Region hin. Das Vas spirale konnte als Blutgefäß nur in 2 Spezimen positiv identifiziert werden und zwar nur in der Spitzenwindung daher scheint seine Funktion als alleinige Ernährungsquelle für das Cortische Organ zweifelhaft.

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ION TRANSPORT IN THE COCHLEA OF GUINEA PIG

II Chloride Transport

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Abstract The Cl^- transport across the endolymph-perilymph barrier in guinea pigs was studied by perfusing the perilymphatic space with artificial perilymph containing ^{36}Cl and measuring the uptake of ^{36}Cl in the endolymph. In normal animals no marked difference in Cl^- concentration was found between the endolymph and perilymph. The data showed that the uptake of ^{36}Cl in the endolymph could be represented by a simple exponential function of the perfusion time, the rate constant being 11.01 min^{-1} . The concentration of ^{36}Cl in the endolymph was greater with perfusion of the scala vestibuli than with perfusion of the scala tympani, indicating that Reissner's membrane is more permeable to Cl^- than the rest of the endolymph-perilymph barrier. Anoxia and local application of ouabain decreased the Cl^- concentration and ^{36}Cl uptake in the endolymph. Our results imply that the endocochlear potential is the principal driving force for unidirectional flux of Cl^- from perilymph to endolymph.

The chloride concentration in the cochlear fluids has been studied by several investigators (in cats, Ledoux, 1950, Citron et al., 1956, in guinea pigs, Citron et al., 1956, Rauch, 1964, Smith et al., 1954, in rats, Bosher & Warren, 1971). These studies indicate that the concentration of Cl^- in the endolymph ranges from 160 to 110 mEq/l and that there is no marked difference in Cl^- concentration between the endolymph, perilymph or cerebrospinal fluid. Recently Bosher et al. (1973) found decreased Cl^- concentration in the endolymph in rats treated with ethacrynic acid and concluded that the observed change in Cl^- concentration must arise from the direct action of ethacrynic acid on the cochlea.

As the endolymph is positively polarized in the resting state, Cl^- will tend to leak into the endolymph. Nevertheless the fact that the

actual concentrations of Cl^- in the endolymph are almost equal suggests the existence of a Cl^- pump which extrudes Cl^- from the endolymph, as has been postulated by Jørgensen (1967). However, extensive studies of Cl^- transport across the endolymph-perilymph barrier have not been available. It is worthwhile investigating the Cl^- transport across the endolymph-perilymph barrier utilizing the radiotracer technique. We are attempting to examine the potential alterations in the Cl^- transport mechanism caused by ototoxic agents.

In the work reported here we studied Cl^- transport across the endolymph-perilymph barrier under the condition of continuous perfusion of the perilymphatic space in the cochlea of guinea pig with artificial perilymph containing ^{36}Cl . Our data suggests that in the resting state the Cl^- concentration in the endolymph is maintained by an outward flux which is energy-dependent and a passive process which is driven by the endocochlear potential (EP).

METHODS

Healthy guinea pigs (NIH strain) were anesthetized with pentobarbital sodium and used throughout our experiments. The methods used for the recording of the cochlear potentials, perfusion of the perilymphatic space and collection of the endolymph and perilymph were the same as those described in the preceding paper (Konishi et al., 1977).

Table 1 ^{-}Cl concentrations in cochlear fluids and EP^a

Perfusate (mM)	Perilymph (mM)		Endolymph (mM)	EP (mV)	n
	Scala vestibuli	Scala tympani			
perfused cochlea	127.8 \pm 5.3	128.6 \pm 5.1	131.0 \pm 5.6	81.3 \pm 2.0	7
unperfused cochlea	138.8 \pm 4.7	139.2 \pm 4.9	138.9 \pm 5.8	83.2 \pm 2.2	5

Values are means \pm standard deviations. n=number of guinea pigs. The scala vestibuli and tympani were perfused for a period of 60 min. Samples were taken at the end of perfusion.

difficult to collect more than one sample of endolymph and perilymph in the scala vestibuli and tympani, separate animals were used for each experiment. An exception to the rule was made in one set of experiments, in which the radioactivity in the perilymph was determined as a function of time in the same animal.

^{36}Cl (3.07 \times 10³ year half life) was obtained as aqueous NaCl solution from Amersham Searle, Illinois. The original solution was diluted with modified Ringer's solution which had the following composition (mM): NaCl 106, KCl 5, Cl₂ 2, NaH₂PO₄ 1, MgCl 1, NaHCO₃ 12, glucose 11. The ^{-}Cl concentration in the perfusate ranged from 160 to 140 mM. The activity of ^{36}Cl in the perfusate was approximately 1 $\mu Ci/ml$. The perfusate was kept at room temperature (24°C). The activities of ^{36}Cl in perfusate, perilymph and endolymph were determined using a liquid scintillation counter. A counting time of 20 minutes was sufficient to produce standard deviation in samples from counting errors to less than 1% of total counts. To determine the ^{-}Cl concentration in the perfusate, perilymph and endolymph, a modification of an electrometric technique developed by Ramsay, et al (1955) was employed. The ^{-}Cl was titrated with the silver ions released by passing a constant current of 100 nA through a silver wire. The current to liberate the silver ions was generated by a solid state constant current injector (Chen and Konishi, 1974). The titration time, defined by the elapsed time between the onset of current application and end point, is linearly proportional to the ^{-}Cl concentration. The sample

volume was kept constant (approximately 10 nl). The standard deviation was less than 1% when replicate samples of 100 mM chloride solution were titrated.

RESULTS

Perfusion of Scala Vestibuli and Tympani

1 Normal guinea pig

The changes in the cochlear responses to sound stimuli observed during perfusion of the perilymphatic space with artificial perilymph containing ^{36}Cl were similar with those observed during perfusion with ^{42}K and ^{22}Na which were described in our preceding paper (Konishi et al, 1978). To summarize briefly, the cochlear microphonics (CM) were only slightly suppressed in a short period of perfusion but gradually decreased with perfusion times longer than 20 minutes (<30% of the initial CM). The action potential of the auditory nerve (AP) and the summating potential (SP) showed temporary changes after the perfusion was initiated but remained stable during the remaining perfusion period. The mean EP recorded at termination of perfusion with varying durations (10 to 90 minutes) was 83.7 \pm 3.8 mV (n=21) which was comparable to 81.3 \pm 2.0 mV (n=7) recorded from the non-perfused cochlea. It was noted that the temporal changes in the cochlear potentials were not affected by variation of the ^{-}Cl concentration in the perfusate ranging from 160 to 140 mM.

The mean ^{-}Cl concentrations in the perilymph of the scala vestibuli and tympani were 127.8 \pm 5.3 mM and 128.6 \pm 5.1 mM respectively in seven non-perfused cochleae (Table

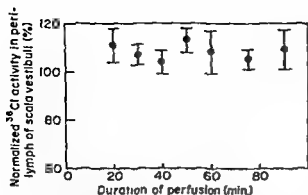


Fig. 1 Mean concentrations of ^{36}Cl in the perilymph of the scala vestibuli for various durations of perfusion. The perfusate was introduced into the scala tympani of the basal turn and both the scala vestibuli and tympani were perfused. The ^{36}Cl concentrations in the scala vestibuli are normalized by taking those in perilymph of the scala tympani as 100%. Vertical range bars indicate standard deviations. Four guinea pigs were used for each perfusion time.

1) The mean ^{36}Cl concentration in the endolymph was 131.0 ± 5.6 mM. No significant differences in the ^{36}Cl concentration were found between the endolymph and perilymph. When the perilymphatic space was perfused for 60 minutes in 5 animals, the mean ^{36}Cl concentration in the perilymph of the scala vestibuli and tympani was 138.8 ± 4.7 mM and 139.2 ± 4.9 mM respectively. The mean ^{36}Cl concentration in the endolymph was 138.9 ± 5.8 mM and the mean EP was 83.2 ± 2.2 mV. Significant differences were observed in the ^{36}Cl concentration when the data from the perfused cochlea were compared with those obtained from the non-perfused cochlea (endolymph to endolymph and perilymph to perilymph, t and u -tests $p=0.05$). However, u - and t tests did not reveal any significant differences ($p=0.05$) in the ^{36}Cl concentration between the endolymph and perilymph in either perfused or non-perfused cochleae.

When the perfusate containing ^{36}Cl was introduced into the scala tympani and both scala vestibuli and tympani were perfused for a period ranging from 10 to 90 minutes, the concentration of ^{36}Cl in the perilymph of the scala vestibuli increased rapidly and reached the level of ^{36}Cl concentration in the perilymph of

the scala tympani 10 minutes after perfusion was initiated. The mean ^{36}Cl concentration in the perilymph of the scala vestibuli remained higher than the ^{36}Cl concentration in the perilymph of the scala tympani. Perfusion was prolonged as shown in Fig. 1.

The ^{36}Cl concentration in the perilymph of the scala vestibuli was found to decrease rapidly in 3 animals after the perfusion was discontinued. The clearance was much faster in the perilymph of the scala tympani than in the perilymph of the scala vestibuli (Fig. 2). The rate of decrease of ^{36}Cl was essentially the same as that of ^{22}Na which was reported in the previous paper (Konishi et al., 1979).

As shown in Fig. 3, the presence of ^{36}Cl in the endolymph could be detected 10 minutes after the perilymphatic space was perfused with ^{36}Cl solution. The concentration of ^{36}Cl in the endolymph increased as the duration of perfusion was prolonged but it did not reach the level of ^{36}Cl concentration in the perilymph. After 60 minutes of perfusion, the ^{36}Cl concentration in the endolymph had reached about 60% of the ^{36}Cl concentration in the perilymph of the scala tympani.

In the steady state, the net flux of ^{36}Cl from the endolymph-perilymph barrier is zero. The concentration of ^{36}Cl in the endolymph

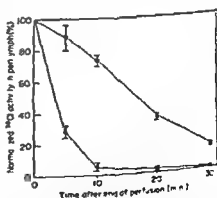


Fig. 2 Clearance of ^{36}Cl after the end of perfusion. \bullet ^{36}Cl concentration in the perilymph of the scala vestibuli; Δ ^{36}Cl concentration in the perilymph of the scala tympani. The ^{36}Cl concentrations are normalized by taking those obtained at the end of perfusion as 100%. Three guinea pigs were used for each time point. Vertical range bars indicate the standard deviation.

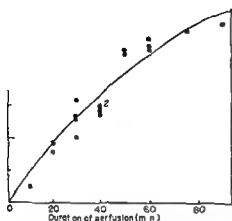


Fig. 3. Uptake of ^{36}Cl in the endolymph as a function of perfusion duration. Both scala vestibuli and tympani were perfused with ^{36}Cl solution. The ^{36}Cl concentration in the endolymph is normalized by taking those in the perilymph of scala tympani as 100%. ● Data points. The line is the least square best fit to the points (see text). Numeral 2 indicates two overlapping data points.

described by the following equation which has been described in detail in the preceding (Konishi et al., 1978)

$$\ln \left(\frac{C_{\text{in}}}{C_{\text{out}}} - \frac{C_{\text{in}}^*}{C_{\text{out}}^*} \right) = \ln \left(\frac{C_{\text{in}}}{C_{\text{out}}} \right) - \frac{J}{V_{\text{in}} C_{\text{in}}} t \quad (1)$$

C_{in} = concentration of ^{36}Cl in endolymph
 C_{out} = concentration of ^{36}Cl in perilymph
 C_{in}^* = concentration of ^{35}Cl in endolymph
 C_{out}^* = concentration of ^{35}Cl in perilymph
 J = Cl flux (mM/min) across the endolymph-perilymph barrier
 V_{in} = volume of endolymph
 t = duration of perfusion

Figure 4 shows the relationship between $\ln \{(C_{\text{in}}/C_{\text{out}}) - (C_{\text{in}}^*/C_{\text{out}}^*)\}$ and duration of perfusion which is expressed by eq. (1). C_{in} is similar to C_{out} and both C_{in} and C_{out} remained unchanged regardless of the duration of perfusion. C_{out}^* is assumed to be constant

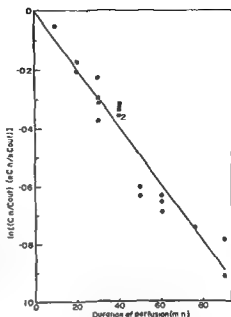


Fig. 4. $\ln \{(C_{\text{in}}/C_{\text{out}}) - (C_{\text{in}}^*/C_{\text{out}}^*)\}$ as a function of perfusion duration. C_{in} and C_{out} represent nonradioactive Cl concentrations in the endolymph and perilymph respectively. They are assumed to be 130 mM. C_{in}^* and C_{out}^* represent radioactive ^{36}Cl concentration in the endolymph and perilymph respectively. The regression line obtained by least square best fit has a slope of 0.01, an intercept of 0.0026, and correlation coefficient is 0.94. Numeral 2 indicates two overlapping points.

because of perfusion of perilymph. By utilizing the least square best fit, the regression line can be expressed by

$$\ln \{(C_{\text{in}}/C_{\text{out}}) - (C_{\text{in}}^*/C_{\text{out}}^*)\} = 0.0026 - 0.01t \quad (2)$$

The data were tested for linearity by using an analysis of variance technique (Dixon & Massey, 1957) and found not to differ significantly ($P=0.05$) from the above line. The transport rate constant was 0.01 min^{-1} and the half time of exchange 69.3 min. The line shown in Fig. 3 was obtained from eq. (2).

2. Effect of anoxia

Intravenous injection of a lethal dose of pentobarbital sodium resulted in a rapid decline of the cochlear potentials including EP. EP changed its polarity and the mean value was -31.9 mV 20 min after perfusion was started.

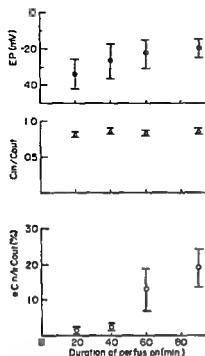


Fig 5 EP (uppermost panel), C_{in}/C_{out} (middle panel) and $*C_{in}/*C_{out}$ (bottom panel) as function of perfusion time in anoxic condition. C and $*C$, total ^{-}Cl concentration and ^{36}Cl concentration respectively, subscript *in* and *out*, endolymph and perilymph respectively. Both scala vestibuli and tympani were perfused after animals received a lethal dose of pentobarbital sodium intrasly. Three animals were used. Vertical range bars are standard deviations.

The EP gradually declined in magnitude as the perfusion proceeded, as shown in the uppermost panel of Fig 5.

The concentration of ^{-}Cl in the endolymph decreased in the anoxic condition. The mean ^{-}Cl concentration ratio of the endolymph to perilymph was 0.84 ($n=3$) with 20 min perfusion and its ratio remained little changed when the duration of perfusion was prolonged to 90 min (middle panel of Fig 5). The reduction of ^{-}Cl concentration in endolymph during anoxia was also observed in the non perfused cochlea and the mean ratio of ^{-}Cl concentration (endolymph to perilymph) was 0.85 ($n=5$) at 40 min anoxic period.

The ^{36}Cl uptake in the endolymph was substantially decreased during the anoxic period. As shown in the bottom panel of Fig 5, with 20 and 40 min perfusion ^{36}Cl uptake in the en-

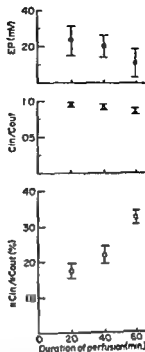


Fig 6 Effect of 10^{-4} M ouabain on EP (uppermost panel), C_{in}/C_{out} (middle panel) and $*C_{in}/*C_{out}$ (bottom panel). Both scala vestibuli and tympani were perfused with ^{36}Cl solution containing 10^{-4} M ouabain. Four animals were used for each perfusion time. See caption to Fig 5.

dolymph was only 1.5 and 2.5% of the concentration in the perilymph. It rose to 19.2% when the duration of perfusion was 60 min.

3 Effect of ouabain

The perfusion of the scala vestibuli and tympani with artificial perilymph containing 10^{-4} M ouabain caused an irreversible suppression of the cochlear responses which was similar to that reported by Konishi & Hamrick (1970). In no case was a negative EP observed with 10^{-4} M ouabain and the rate of cessation of the EP was generally slower than observed in anoxia (uppermost panel of Fig 6). The mean ^{-}Cl concentration ratio (endolymph to perilymph) did not show a significant reduction and was about 0.95 with 20 min perfusion ($n=4$). A slight decline of the ratio was noted as the perfusion was prolonged. The ^{36}Cl concentration in the endolymph with 20 min perfusion was 18.2%

· II Distribution of ^{36}Cl in perilymph and endolymph and EP under different conditions of perfusion in normal guinea pigs^a

perfusion		Perilymph (%)		Endolymph (%)	EP (mV)	n
α	Cold sol	Scala vestibuli	Scala tympani			
IT		100.8 ± 7.8	100	28.6 ± 1.2	82.4 ± 2.3	5
		100	2.0 ± 0.2	28.3 ± 0.6	79.6 ± 1.5	3
		90.7 ± 14.5	100	17.7 ± 0.6	79.3 ± 1.6	3
		100	0.5 ± 0.3	33.0 ± 1.8	81.0 ± 1.7	3
	ST					
	SV	3.7 ± 2.7	100	4.2 ± 1.0	82.7 ± 2.2	4

Values are means ± standard deviations. n = number of guinea pigs. Hot solution and cold solution. artificial perilymph and without ^{36}Cl respectively. Duration of perfusion 40 minutes. ^{36}Cl concentrations are normalized to perilymph scala to which hot solution was introduced.

to that in the normal condition but with perfusion longer than 20 min suppression of uptake in the endolymph was noticeable. At 60 minutes perfusion the mean ^{36}Cl concentration in the endolymph was $35.0 \pm 3.1\%$ that in the perilymph which was about 20% than the mean value obtained in normal animals.

Perfusion of Scala Tympani vs Scala Vestibuli

Temporal changes in the cochlear potentials observed during perfusion of the scala tympani alone were similar to those observed during perfusion of both scala vestibuli and scala tympani. The changes in cochlear potentials associated with perfusion of the scala vestibuli were in most cases less than those observed during perfusion of the scala tympani as described in the previous section. The mean ^{36}Cl concentration in the perilymph of the scala vestibuli was slightly higher than that in the perilymph of the scala tympani when both the scala vestibuli and tympani were perfused for 40 min by introducing the perfusate into the scala tympani of the basal turn. The mean ^{36}Cl concentration in the endolymph was $6.1 \pm 2\%$ ($n=5$) of that in the perilymph of the scala tympani (Table II). The perfusion of the scala vestibuli alone for a period of 40 min resulted in a mean ^{36}Cl concentration in the endolymph of $28.3 \pm 0.6\%$ ($n=3$) which was comparable to that found in perfusion of both

scala vestibuli and tympani. However the ^{36}Cl concentration in the perilymph of the scala tympani was significantly lower ($2.0 \pm 0.2\%$). The low activities of ^{36}Cl in the perilymph of the scala tympani are probably due to the rapid clearance of ^{36}Cl in the scala tympani as shown in Fig. 2. The perfusate introduced into the scala vestibuli reaches the scala tympani through the spiral ligament but the clearance of ^{36}Cl is so fast in the scala tympani that no increase in its concentration could be observed. When the site of the perfusion was switched from the scala vestibuli to the scala tympani, the mean ^{36}Cl concentration in the endolymph was $15.7 \pm 0.6\%$ ($n=3$) but the mean concentration in the perilymph of the scala vestibuli was $90.7 \pm 14.5\%$ relative to the perilymph in the scala tympani.

The results obtained in the separate perfusion of the scala vestibuli or scala tympani were substantiated by a dual perfusion, simultaneous perfusion of the scala vestibuli with ^{36}Cl -Ringer's and perfusion of the scala tympani with nonradioactive Ringer's or vice versa. When the scala vestibuli was perfused with ^{36}Cl -solution and simultaneously the scala tympani was perfused with Ringer's for 40 min, the mean EP at the time of endolymph collection was 81.0 ± 1.7 mV ($n=3$). The mean ^{36}Cl concentration in the endolymph was $33.0 \pm 1.8\%$ of that in the perilymph of the scala vestibuli. The ^{36}Cl concentration was, of course, very low in the samples taken from

the scala tympani. When the dual perfusion was carried out in the reverse manner, namely perfusion of the scala tympani with ^{36}Cl solution and the scala vestibuli with non-radioactive solution for 40 min, the mean EP was $82 \pm 2.2 \text{ mV}$ ($n=4$) and the ^{36}Cl concentration in the endolymph was substantially lower with the mean value of $4 \pm 1.0\%$ than that in the perilymph of the scala tympani.

DISCUSSION

Our results demonstrate that the mean concentration of ^{36}Cl in the endolymph and perilymph are 131 and 128 mM respectively and that there is no marked concentration gradient across the endolymph-perilymph barrier in the non-perfused cochlea. Our findings are in line with the previous reports (Smith, 1954, Rauch, 1964, Citron et al., 1956, Ledoux, 1950 and Bosher & Warren, 1971). The mean concentration of ^{36}Cl in the endolymph and perilymph as increased to 139.0 and 138.9 mM respectively by perfusing the perilymphatic space with the artificial perilymph containing ^{36}Cl concentration ranging from 140 to 160 mM. However, it was noted that the perfusion of the perilymphatic space did not create a ^{36}Cl concentration gradient across the endolymph-perilymph barrier. In addition, perfusion did not result in marked suppression of the cochlear potentials. It is safe to assume that the introduction of perfusate with ^{36}Cl concentration ranging from 140 to 160 mM into the perilymphatic space does not greatly alter the physico-chemical condition of the cochlea and does not modify the ^{36}Cl transport process.

Sellick & Johnstone (1975) cited the unpublished data of Robinson & Sellick, in which the scala media was perfused with low Cl sulfate Ringer's, producing an increase in EP of about 10 mV. However, the equilibrium potential for ^{36}Cl changed from 0 to -92 mV (with 3 mM ^{36}Cl concentration in the endolymph). The small effect on EP led them to assume that

Reissner's membrane and other parts of the cochlear partition are relatively impermeable to ^{36}Cl . Kuypers (1969) concluded the significant contribution of ^{36}Cl to EP, which concluded on the basis of his experiments which showed that a deficiency of Cl in perilymph affected EP to only a slight extent. The rate constant for ^{36}Cl calculated from the results was found to be 0.01 min^{-1} and is much smaller than the rate constant for K^+ (Kishi et al., 1978). Since the flux (J) is the product of volume, concentration and rate (see eq. 1), the ratio of flux of Cl to that of K^+ is found to be 0.67, when ^{36}K and Cl concentration in the endolymph is assumed to be 130 and 130 mM respectively. Similar calculations show that the flux of ^{36}Cl across the lymph-perilymph barrier is much greater than the flux of ^{22}Na ; the flux ratio of Cl to Na is 60 to 90 (^{22}Na concentration is assumed to be 1 to 2 mM).

The results reported here show that the concentration of ^{36}Cl in the endolymph is greater in the scala vestibuli than in the scala tympani. Therefore it is likely that Reissner's membrane is more permeable to ^{36}Cl than the rest of the cochlear partition. One may argue the possibility of rupture of Reissner's membrane during the course of perfusion or collection of endolymph or perilymph in the scala vestibuli. If this is the case, it may be expected that the observed uptake of ^{36}Cl in the endolymph will be higher, because the endolymph would be contaminated by perfusate. This possibility can be ruled out by the fact that the cochlear responses to acoustic stimulation decrease progressively during the perfusion and the EP recorded at the end of the endolymph collection showed a slight decrease ranging between 80 and 90 mV. Therefore, the results imply that Reissner's membrane is more permeable to ^{36}Cl than the rest of the cochlear partition. Reissner's membrane investigation carried out by Chou (1970) showed a remarkable consumption of oxygen by Reissner's membrane. Autoradiographic investigation of Reissner's membrane showed that

poration of leucine (Plester, 1960) Ilberg (1961) injected a colloidal suspension of thorium dioxide into the perilymphatic or endolymphatic space and found that thorium dioxide crossed Reissner's membrane in both directions, by a combination of diffusion and an active transport mechanism. Hinojosa (1971) reported that transport of ferritin takes place across Reissner's membrane from the perilymph toward the endolymph but not in the opposite direction. The mechanism underlying transport across Reissner's membrane is still a matter of speculation in the present state of our knowledge. Further studies are needed to clarify the Cl^- transport mechanism across Reissner's membrane.

There was no marked Cl^- concentration difference between the endolymph and perilymph in both non-perfused and perfused cochleae, the equilibrium potential due to Cl^- derived from the Nernst equation is roughly equal to zero. From the fact that the resting potential of EP ranges from 80 to 90 mV, it is obvious that Cl^- is not passively distributed in cochlear fluids. It is conceivable from our results that the EP is a major driving force for the inward movement of Cl^- from the perilymph to endolymph. In anoxic conditions the magnitude of the EP decreases rapidly and usually the polarity of EP changes to negative within a few minutes after anoxia is induced. The negative EP repels the Cl^- ions in the endolymph toward the perilymph, reducing the inward flux of Cl^- from the perilymph to endolymph. This accounts for a decrease in Cl^- concentration in the endolymph. Instillation of 10^{-4} M ouabain into the perilymph resulted in suppression of CM and AP, and it reduced the magnitude of EP. At the 10^{-4} M ouabain concentration a negative EP was not observed in our experiments. The flux of $^{36}\text{Cl}^-$ from the perilymph to endolymph may be affected by the slowly declining EP produced by 10^{-4} M ouabain to a lesser extent than by the rapidly declining EP produced by anoxia.

In order to maintain the balance of Cl^- concentration between the endolymph and peri-

lymph, it is apparent that Cl^- in the endolymph must be extruded from the endolymph. Johnstone (1971) postulated several types of Cl^- transport mechanism and presented assumptions that ^+K may be pumped in by an electrogenic pump and ^+Na and Cl^- may be pumped by an electrically neutral pump. The data presented here do not provide any conclusive evidence concerning the mode or site of the outward directed Cl^- pump. The rate of ion transport is generally determined by the three factors, active transport, passive transport and solvent drag (Ullrich, 1974). In the steady state the net flux is zero and if the inward Cl^- flux from the perilymph to endolymph is assumed to be passive, then the outward flux is the sum of active transport and solvent drag. It has been noted that solvent drag across epithelial tissues has been demonstrated convincingly only for relatively small, uncharged solutes (Leaf & Hays, 1962). At present it is difficult to evaluate precisely the physiological importance of solvent drag as a transport mechanism in the cochlea. The solvent drag may represent a potentially important transport process that is as yet unexplored in the cochlea. Further studies along this line are needed before the outward flux of Cl^- can be precisely evaluated.

The dynamic equilibrium of electrolytes in the cochlear fluids is indispensable for maintaining the high sensitivity of the hair cells of the organ of Corti. The understanding of the mechanism of homeostasis in the cochlear fluids is essential in studying the pathogenesis of various hearing impairments that may occur as a result of disturbance of the dynamic equilibrium of electrolytes in the cochlear fluids. The results described here demonstrate our first step to elucidate the mechanism of electrolyte exchange between the perilymph and endolymph. Our results indicate that the endolymph-perilymph barrier—especially Reissner's membrane—is permeable to Cl^- and the inward flux of Cl^- across the endolymph-perilymph barrier is passive, mainly driven by EP. The Cl^- in the endolymph may be extruded

from the endolymph by an active transport mechanism or other process such as solvent drag

ZUSAMMENFASSUNG

Die Cl^- Überführung durch die Schranke der Endolymph-Perilymphe wurde bei Meerschweinchen durch Perfundieren des perilymphatischen Raumes mit künstlicher Perilymphe die ^{36}Cl -thielt und Messung ^{36}Cl Aufnahme in die Endolymph untersucht. Bei normalen Tieren wurde kein bemerkenswerter Unterschied in Cl^- Konzentration zwischen Endolymph und Perilymphe festgestellt. Die Befunde zeigten, daß die ^{36}Cl Aufnahme in die Endolymph als einfache exponentielle Funktion der Perfundierungszeit darstellbar ist mit einer Geschwindigkeitskonstanten von 0.61 min^{-1} . Die Konzentration von ^{36}Cl in der Endolymph war höher, wenn die Scala vestibuli perfundiert wurde, als wenn die Scala tympani perfundiert wurde, was andeutet, daß die Membrana vestibularis für Cl^- mehr durchlässig ist als die übrige Schranke der Endolymph-Perilymphe. Anoxie und örtliche Anwendung von Ouabain reduzierten die Konzentration von Cl^- und die Aufnahme von ^{36}Cl in die Endolymph. Unsere Ergebnisse lassen darauf schließen, daß das endocochleare Potential die hauptsächlichste Antriebskraft für den einseitig gerichteten ^{36}Cl Fluß von der Perilymphe zur Endolymph liefert.

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OBSERVATIONS OF OTOCONIAL MEMBRANES
FROM HUMAN INFANTSCharles G Wright¹ and David G Hubbard²*From the ¹Callier Center for Communication Disorders The University of Texas at Dallas
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The microdissection technique was used to otoconial membranes from 30 human infants ranging newborn to 2 years of age. Both saccular and utric membranes were quite variable in overall shape. In the neonatal period the gelatinous layer of the membrane appears to thicken and become less to the macular surface than in the fetal period. In many infants older than 6 weeks otoconial membranes found at autopsy to be completely dislodged from maculae with abnormally large saccular otoconia in four specimens. Fourteen of the infants studied of sudden infant death syndrome and 16 died of other causes. The incidence of detached otoconial membranes was approximately the same in both groups. Although the majority of these membranes were apparently dislodged post mortem the present findings suggest that otoconial membranes are susceptible to pathological alteration due to disease or head trauma.

In recent studies on inner ear pathology man have dealt with abnormalities of the otoconial membranes which cover the saccular and utricular maculae. Johnsson & Hawkins (1971, 1972) have, for example, demonstrated a loss of both saccular and utricular otoconia in patients over 60 years of age. Ross et al (1976) utilized the scanning electron microscope to study degenerative changes in otoconia from infants as well as from older individuals. In addition, Schuknecht (1974) provided evidence that dislodged otoconia enter the posterior ampulla and provoke a disorder termed 'cupulolithiasis' which is characterized by positional vertigo of the benign paroxysmal type. The study of human otoconia has been facilitated by the microdissection technique (Hawkins & Johnsson 1975) which allows otoconial membranes to be examined in

tact and avoids decalcification procedures which radically alter the otoconial crystals.

Accurate assessment of pathological alterations in human otoconial membranes depends upon recognition of artifactual changes which may occur during the postmortem period or during histological processing. Knowledge of the normal range of morphological variation in these structures is also of importance in this regard. Such data are, at present, quite limited.

Our study is part of a continuing investigation of macular suprastructures in man dealing with the development of otoconial membranes, as well as their possible involvement in inner ear pathology. This report focuses upon observations in human infants up to 2 years of age.

METHODS

Inner ears from a total of 60 individuals were studied during the course of this investigation. Thirty of these cases were fetuses obtained from premature deliveries which occurred during the third trimester of pregnancy or from prostaglandin abortions induced in the second trimester. The other 30 cases were infants ranging from newborn to 2 years of age.

Temporal bone specimens from the infants were removed at autopsy using a bone plug saw one inch in diameter. Fetal specimens were cut from the skull floor with heavy surgical scissors. The inner ears were fixed by perilymphatic perfusion utilizing either 1%

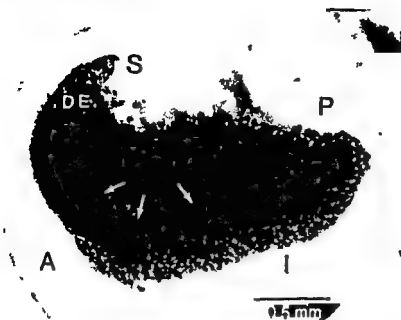


Fig. 1. Surface view of a saccular otoconial membrane from a 9-month-old infant. Arrows indicate "snowdrift" line. DE denotes direction of dissection. Anatomical orientation of the skull indicated by S (superior), A (anterior), P (posterior), I (inferior), and L (lateral).

osmium tetroxide or 2% glutaraldehyde. Both fixatives were veronal-buffered at pH 7.4. When glutaraldehyde was used as the primary fixative the inner ear structures were stained with 1% osmium tetroxide following overnight glutaraldehyde fixation at refrigerator temperature. After staining, specimens were partially dehydrated in ethanol and studied promptly to avoid possible artifacts due to prolonged storage.

With minor modifications, all material was prepared for study by the microdissection technique as described by Hawkins & Johnson (1975). The vestibule was opened by enlargement of the oval window and the saccular otoconial membrane photographed *in situ* with a Wild-Heerbrugg Model 7S stereomicroscope. The anterior portion of the utricle containing the macula utriculi was then carefully dissected out and trimmed with insectomy scissors to allow examination and photography of the utricular otoconial membrane.

For scanning electron microscopy, otoconial membranes were removed from the maculae and placed on aluminum specimen holders. They were then air-dried over silica gel, sputter-coated with gold and studied with

a JEOL model 35 scanning electron microscope operated at 15 kV.

RESULTS

The surface morphology of the saccular otoconial membrane is illustrated in Fig. 1. The specimen from a 9-month-old infant conforms with the shape of the sacculus; the otoconial mass is roughly oval with an extension at its anterior end which projects dorsally. A curved ridge known as the "snowdrift" line (Ades & Engstrom, 1966) is a prominent feature of the crystal layer. This band in the otoconial membrane underlies the striae of the sensory epithelium (Ades, 1969).

A distinct "snowdrift" line is also present on the surface of the utricular otoconial membrane as can be seen in Fig. 2 with a specimen from a 16-month-old infant.

Both the saccular and utricular otoconial membranes of infants included in this study vary considerably in overall shape between individuals. As illustrated in Fig. 1 the



Fig 2 Utricular otoconial membrane from a 16-month-old female. Snowdrift line is indicated by arrows. A anterior, P posterior, L lateral, M medial.

ions were particularly striking in saccular membranes, due largely to differences in configuration of the dorsal extension. Otoconial membranes from fetuses and newborns were found to be tightly adherent to the macular surface and therefore were very difficult to dissect away from the sensory epithelium intact. In these specimens, the gelatinous layer underlying the otoconia appeared to be thinner and less cohesive than in older individuals. Otoconial membranes from infants older than about 8 weeks could be dis-

placed off the maculae more easily and they were perceptibly firmer in texture, even though they had been processed in the same way as those from fetuses and younger infants. Altogether, 59 temporal bones from 30 infants were examined in the present study. In 10 of these the otoconial membranes were found to be displaced from their normal position in the saccule and/or utricle. In each of these cases it appeared that the membrane had slipped from the macular area and crum-

pled into an irregularly shaped mass which was found lying unattached inside the saccule or utricle. Typical examples of such detached otoconial membranes are shown in Fig 4.

This phenomenon is definitely not due to fixation or storage artifact since it is possible to see the saccular macula clearly through the oval window and evaluate the condition of the otoconial membrane before fixative solution is introduced into the labyrinth. It is usually also possible in unstained specimens to determine whether the utricular membrane is in place over the sensory epithelium.

In four of the inner ears in which displaced membranes were present, the saccular otoconial mass contained very large crystals such as those illustrated in the scanning electron micrographs of Fig 5. Normal saccular otoconia from the infants included in this investigation were approx. 4–15 μm in length, those from the membranes containing large crystals were up to 65 μm long and sometimes had abnormal shapes. No abnormalities were found

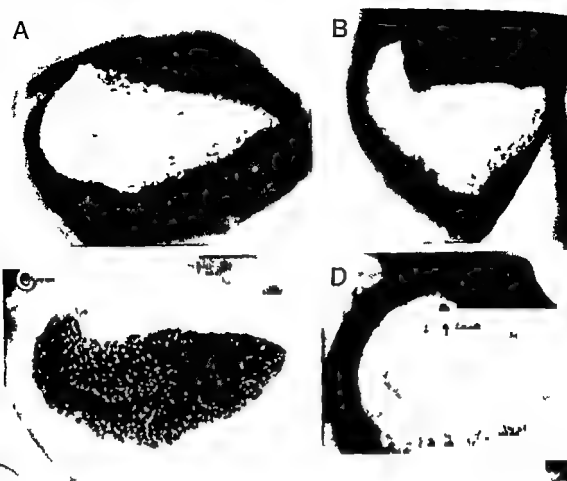


Fig. 3. Variations in overall shape of saccular otoconal membranes from infants 3 months (A), 5 months (B), 11 months (C) and 24 months (D) of age.

in the utricular otoconia from these specimens.

Of the 30 infants whose inner ears we studied, 14 were victims of sudden infant death syndrome (SIDS) and 16 died of readily identifiable causes. In both these groups middle ear effusions were frequently found at autopsy. Specifically, 14 (52%) of the 27 SIDS specimens examined had effusions and 12 (38%) of the 32 temporal bones from infants who died of known causes were found to have middle ear effusions. The middle ear mucosa was markedly hyperemic and edematous in all these cases regardless of the type of effusion present [clear fluid (serous), viscous, yellowish mucous (mucoid) or pus (purulent)].

Specific data regarding the condition of oto-

conial membranes, presence of middle ear effusions, age and approximate post-mortem time before fixation are given in Tables I and III.

Since our observations on neonatal conial membranes suggest that they have physical characteristics much like those of fetuses, the data on infants up to 6 weeks of age are presented separately in Table I. Attached membranes were present in 2 (11%) of the 16 specimens in this group. Prevalence is similar to that found in several third trimester fetuses: dislodged membranes were present in 6 of the 60 ears in our collection.

As Tables II and III show, dislodged membranes were found with much greater

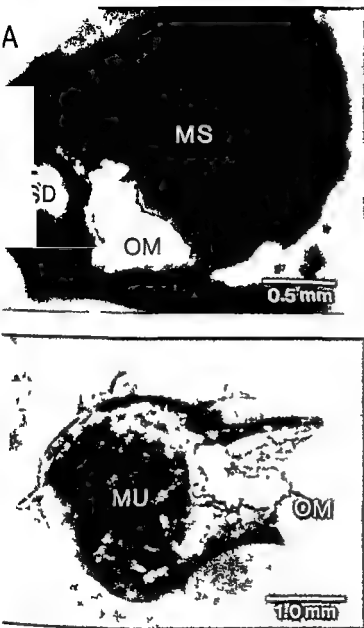


Fig 4 (A) Displaced crumpled saccular otoconial membrane (OM) from a 7 month-old infant. The letters MS are placed in the central portion of the darkly stained saccular macula. SD, saccular duct. (B) Utricular macula (MU) from the same infant showing detached otoconial membrane (OM) lying near medial aspect of macula. Note difference in scale relative to (A).

frequency in older infants. Also, the incidence was approximately the same in SIDS victims as in children who died of other causes: 70% in the SIDS group and 75% in the other cases. We believe this finding is related to a developmental change occurring during the first several weeks of life which renders the otoconial membrane more susceptible to displacement. The actual detachment of membranes from

the macular areas may be caused by a variety of factors, some of which will be considered in the following discussion.

DISCUSSION

The present investigation of inner ears from human infants revealed marked variations in the shape of otoconial membranes between



Fig. 5 (A) Normal saccular otoconia from a 2-year-old female infant (Case 44) (right). (B) Giant saccular otoconia from a 6-month-old infant (Case 37). The largest crystals seen in this view are approximately 40 μm long. Otoconia up to 65 μm in length were present in other areas of the

specimen. (C) Very large saccular otoconia from a 1-month-old infant (Case 24). Note dense, fibrous-like packing and peculiar shape of some crystals. The indication (indicated by bar at lower right) is the same in each micrograph.

Table I Data on infants up to 6 weeks of age

1st column identifies major pathology '+' indicates intact otoconial membrane, '0' detached membrane Abbreviations: PMT, post mortem time, S, saccule, U, utricle, R, right, L, left, Bilat., bilateral

Case	Age	PMT (hours)	Right ear		Left ear		Middle ear effusion
			S	U	S	U	
SIDS	6 weeks	6	+	+	+	+	R, none, L, serous
SIDS	6 weeks	21	0	0	+	+	Bilat. serous
Sepsis	1 week	15	+	+	+	+	None
Coarctation of aorta	1 day	17	+	+	+	0	None
Hydrocephalus	newborn	14	+	+	+	+	None
Omphalocele	11 weeks	8	+	+	+	+	None
Aortic atresia	4 days	24	+	+	+	+	None
Coarctation of aorta	6 weeks	20	+	+	+	+	None

Table II Data on infants who died of causes other than SIDS

Major pathology identified in first column Symbols and abbreviations as in Table I

Case	Age (months)	PMT (hours)	Right ear		Left ear		Middle ear effusion
			S	U	S	U	
Sepsis	16	8	+	+	+	0	None
Encephalitis	4	11	0	0	0	0	R, mucous, L, purulent
Cerebral hemorrhage	11	8	+	0	+	0	Bilateral purulent
Asphyxiation	2	4	0	0	+	+	Bilateral serous
Sepsis	18	22	0	0	0	0	None
Burn victim	24	8	+	+	0	0	None
Asphyxiation	5	14	0	0	0	0	None
Asphyxiation	4	13	+	+	+	+	R, mucous, L, purulent
Asphyxiation	7	12	0	0	0	0	Bilateral purulent
Fatty liver	3.5	4	0	+	0	+	R, mucous, L, purulent

Table III Data on SIDS victims

Symbols and abbreviations as in Table I Only the left ear from Case 2 was available for study

Case	Age (months)	PMT (hours)	Right ear		Left ear		Middle ear effusion
			S	U	S	U	
1	3	10			0	+	None
1	2	5	0	0	0	0	None
4	11	12	0	0	0	0	Bilat. purulent
5	2	6	0	0	+	0	None
7	4	6	+	+	+	0	None
0	3	8	+	+	+	+	R. purulent, L., none
2	9	19	0	0	+	0	Bilat. purulent
3	3	12	+	+	0	0	None
5	2	10	0	0	0	0	Bilat. serous
6	3	6	+	+	+	+	None
7	11	21	0	0	0	0	Bilat. purulent
8	5	10	0	0	+	+	Bilat. purulent

different individuals. This variability is particularly striking in saccular membranes where obvious differences in the size and shape of the dorsal extension are readily observable.

In common with fetal otoconial membranes, those from infants up to about 6 weeks of age were firmly adherent to the macular surface. Otoconial membranes from older infants appeared to have a thicker, more cohesive gelatinous layer. They were much easier to dissect away from the maculae intact.

This maturational change in the physical character of the otoconial mass is apparently of significance in accounting for our finding that many otoconial membranes in older infants were completely detached from the vestibular maculae at the time they were first examined, before the inner ears were perfused with fixative.

Fourteen of the 30 infants whose temporal bones were included in this study were victims of sudden infant death syndrome, in the remaining cases death was attributable to accidental causes or to specific pathological lesions. Dislodged otoconial membranes were found with about equal frequency in both these groups of infants.

In accordance with previous studies of neonates and infants (McLellan et al, 1962, Paradise et al, 1976), we noted a rather high incidence of middle ear effusions in the present material. Effusions were found about 14% more frequently in the SIDS group. This is not particularly surprising in view of the fact that mild infections, especially of the upper respiratory tract, have been reported in a high percentage of SIDS victims (Naeye, 1973, Naeye et al, 1976).

Arnold et al (1977) have provided evidence that chronic mucous effusions in children can produce a sensorineural hearing loss which they believe is due to a reduction in oxygen supply to the inner ear and to the influence of histamine released by the metaplastic epithelium of the middle ear. An increased incidence of sensorineural hearing loss in patients with chronic purulent otitis media is also

well documented (Paparella et al 1970). regard to the vestibular end organs knecht (1974) has noted a positive correlation between the presence of otitis media and disorder known as cupulolithiasis which believed to involve dislodged otoconia. It seems possible that biochemical alterations in the inner ear fluids (associated with middle ear effusions) might produce pathological changes in the otoconial mass in infants.

It is interesting to note at this point that each of the three cases in our series of giant saccular otoconia were found when otitis media was also present. The large otoconia were definitely not artifacts due to fixation or storage and they seem unlikely to be the result of post mortem autolysis since the utricular otoconia were normal in these infants. In relation to the specific effects of autolysis on the otolith organs the work of Ross et al (1976) is also pertinent. Using Rhesus monkeys in experimental studies, they found that the otoconial membranes remained intact and contained normal otoconia after post mortem times as long as 72 h.

On the basis of data collected thus far, however, there appears to be little evidence that middle ear effusions contribute significantly to the actual displacement of otoconial membranes. Among the cases listed in Table I, the incidence of detached membranes is the same in those with middle ear effusions as in those free of effusions. In the SIDS group (Table III), 9 of the 11 temporal bones with effusions also had dislodged membranes while in 7 of 12 ears without effusions displaced otoconial membranes were found.

Certainly, one of the greatest difficulties in the study of anatomical materials obtained at autopsy is the evaluation of effects produced by post mortem autolysis. In the present study this is especially true of specimens from SIDS cases since only a rough approximation of the time delay between death and fixation of the temporal bones can be made.

We can, however, divide these cases into two subgroups on the basis of relative

at mortem time. If this is done, it is found that displaced otoconial membranes were in 5 (50%) of the 10 ears with post-mortem times of less than 10 hours. At long post-mortem times the incidence is considerably higher: 11 (85%) of the 13 SIDS specimens with post-mortem times of 10 hours or more had dislodged membranes. The data from infants who died of other causes shows an incidence of 70% at post-mortem times between 10 hours and 80% at 10 hours or more. Thus in both groups of infants, detached otoconial membranes were found more frequently in material in which the post-mortem time was long. As Table I shows, the 2 younger infants with dislodged membranes also had post-mortem times close to 20 hours. It should be noted, however, that in some instances (e.g. case 21) all four membranes were detached even though the post-mortem time was relatively short and in others (such as case 1) the otoconial membranes were intact in spite of long post-mortem times.

Finally, vibration of temporal bone specimens by the oscillating saw used during excision from the skull may dislodge the otoconial mass in some cases. Interestingly enough, vibration from the saw was evidently often severe enough to dislodge membranes in infants less than 6 weeks old. Also, vibration cannot be the sole cause for membrane displacement since detached membranes were occasionally found in fetal specimens which were removed from the skull with scissors.

In conclusion, our observations suggest a nuance of developmental change in human otoconial membranes after birth. During the neonatal period, the gelatinous layer of the otoconial mass appears to thicken and separate from the macular surface to the extent that the entire membrane may be dislodged rather easily from the macula. Approximately three-quarters of the 43 ears studied in infants older than 6 weeks contained saccular and/or utricular otoconial membranes which were completely detached from the maculae.

Complete membrane detachment apparently occurs during the post-mortem period in most cases. This phenomenon is therefore not necessarily indicative of pathology. Otoconial membranes may be dislodged as a consequence of mechanical vibration during autopsy procedures, or as the result of autolysis when the delay before fixation of specimens is long.

On the other hand, the results of this study do suggest that otoconial membranes are susceptible to pathological involvement in the presence of ear disease or head trauma. This is particularly true for older infants in whom the membranes are more loosely attached to the sensory epithelia. These findings therefore point toward a need for reassessment of the more traditional concept in which the macular suprastructures have been considered to be static and invariable after the time of birth.

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ZUSAMMENFASSUNG

Mittels Mikrosezieren sind die Otolithenmembrane von 30 Kleinkindern im Alter von neugeborenen bis zwei Jahre alt untersucht worden. Sowohl die Sacculusmembran als auch die Utriculusmembrane waren sehr unterschiedlich in ihrer Form. Im Vergleich zur Fetusphase scheint es, daß in der Neugeborenenphase die gelatinöse Schicht der Otolithenmembran sich verdickt und sich etwas von der Maculafläche löst. Bei vielen Säuglingen, die älter als sechs Wochen waren, zeigte die Autopsie, daß die Otolithenmembrane sich gänzlich von der Macula gelöst hatten und in vier Fällen fand man anormal große Sacculusotolithenchristalle. Vierzehn der untersuchten Säuglinge starben an dem plötzlichen infant death syndrome (plötzliches Säuglingssterbesyndrom) und sechs starben durch andere Ursachen. Die Zahl der gelösten Otolithenmembrane war für beide Gruppen fast gleich groß. Obwohl die Mehrzahl dieser Membrane sich scheinbar erst postmortem gelöst hatten, lassen die bis jetzt vorliegenden Untersuchungsergebnisse vermuten, daß bei Krankheiten oder Kopftrauma die Otolithenmembran zu pathologischen Veränderungen neigt.

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FURTHER STUDIES OF THE EFFECTS OF CONTINUOUS WHITE NOISE OF MODERATE INTENSITY (70-80 dB SPL) ON THE COCHLEA IN YOUNG GUINEA PIGS

Time Course and Distribution of Hair Cell Degeneration

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Abstract Guinea pigs aged one week were exposed to white noise at a maximum of 76 dB SPL for 7 days and were then killed 3, 8 and 16 weeks later for histological examination of the cochlea by the surface preparation method. Appreciable increases in outer hair cell losses were observed in the apical turn 3/3, chiefly in the outer rows between the 3rd and 8th week but not between the 8th and 16th week. No significant losses were seen in control groups corresponding to the 3 and 8 week periods although in the control group for 16 weeks survival small deficits attributable to natural ageing were seen in the apical half turn 3/3.

The question of whether exposure to excessive hospital incubator sound is likely to cause losses in premature babies has been the subject of some discussion (e.g. Seleny & Ryan, 1969; Blennow et al., 1974; Falk et al., 1974). It is well established that young animals are, in general, more susceptible than old to high intensities, that is, about 120 dB (e.g. Falk et al., 1974; Coleman, 1976b), and recently we have shown that continuous white noise as low as 70-80 dB, an amplitude quite low in hospital incubators, can cause considerable damage to the cochleas of very young guinea pigs (Douek et al., 1976). In our study we assessed cochlear damage by the surface preparation method (Engstrom et al., 1976) allowing 3 weeks to elapse between the end of acoustic stimulation and histological examination to permit damaged hair cells to degenerate. However, in a later electron microscope study of the process of degeneration (Dodson et al., 1977) we have found evi-

dence that degeneration of hair cells continues well beyond 3 weeks after experimental stimulation, suggesting that the final level of damage might be considerably greater than that seen in our previous study. Similar long term effects have also been detected electron microscopically by Spoendlin & Brun (1973), although little is known about the quantitative aspects of such changes or indeed about the time course of hair cell degeneration after any type of trauma.

It was therefore decided to determine the final extent of hair cell loss in young guinea pigs, after a continuous, 1 week white noise exposure and to establish the characteristic pattern of deficits in the different regions of the cochlea. In addition, since the number of lost hair cells was not great in our previous study, it could be objected that such deficits might be caused by uncontrolled noise in the animal house in which the guinea pigs were kept, or, in the long term experiments, they might be caused by natural ageing processes; accordingly, further controls have been carried out to evaluate the contribution of such factors to the observed damage.

MATERIALS AND METHODS

Groups of guinea pigs (Dunkin Hartley strain) aged 1 week were placed with their mothers in an acoustically insulated box fitted with two

Table 1 An analysis of outer hair cell losses in experimental and control groups in this investigation

On the left the cochlear half turns of animals surviving for 3, 8 and 16 weeks after treatment are compared, on the right a similar comparison of the control groups of corresponding ages is shown

Abbreviations: NS, not statistically significant; OHC, outer hair cell

Experimental groups				Control groups		
Cochlear region	Survival (weeks)	Mean and S.D. (% OHC loss)	P	Age (weeks)	Mean and S.D. (% OHC loss)	P
31	3	10.4 ± 6.0	<0.05>0.02	5	1.1 ± 0.9	NS
	8	18.7 ± 7.5		10	0.8 ± 0.7	<0.01
	16	16.7 ± 10.7	NS	18	5.6 ± 1.1	
3	3	3.6 ± 3.3	<0.02>0.01	5	0.8 ± 0.7	NS
	8	12.0 ± 7.2		10	1.3 ± 0.8	<0.1>0.01
	16	15.4 ± 11.2	NS	18	3.4 ± 1.6	
21	3	2.3 ± 3.1	NS	5	0.4 ± 0.4	NS
	8	3.0 ± 2.4	NS	10	1.2 ± 0.6	NS
	16	10.1 ± 11.4		18	2.3 ± 1.3	
2	3	2.6 ± 2.6	NS	5	0.3 ± 0.4	NS
	8	0.5 ± 0.4	<0.05>0.02	10	0.3 ± 0.3	NS
	16	1.0 ± 0.4		18	0.4 ± 0.3	
11	3	1.4 ± 1.5	NS	5	0.6 ± 0.7	NS
	8	0.2 ± 0.2	<0.01>0.001	10	0.6 ± 0.7	NS
	16	1.5 ± 0.6		18	0.7 ± 0.9	
1	3	1.9 ± 4.5	NS	5	0.5 ± 0.8	NS
	8	0.1 ± 0.1	NS	10	0.1 ± 0.1	NS
	16	0.2 ± 0.5		18	2.4 ± 5.0	
1	3	0.1 ± 0.1	NS	5	0.1 ± 0.1	NS
	8	0.1 ± 0.1	NS	10	0.0 ± 0.0	NS
	16	0.1 ± 0.1		18	0.4 ± 0.6	

loudspeakers connected to a white noise generator, and were subjected to continuous white noise for 7 days. A frequency spectrograph of the acoustic stimulus, measured with a Bruel & Kjaer sound level meter inside the box, is shown in Fig. 1. It will be noted that there are two maxima, one at 500 Hz and the other at 4000 Hz, both of 76 dB.

After acoustic stimulation, animals were kept for 3, 8 or 16 weeks and were then killed with an overdose of barbiturate, decapitated and their cochleas fixed in 3% phosphate buffered glutaraldehyde (pH 7.4, 0.1 M) for 4 hours, small holes being made in the cochlear bone to admit the fixative. The cochleas were then washed in buffer, postfixed in buffered 1% osmium tetroxide and partially dehydrated in 70% ethanol. Pieces of spiral organ were dissected out, then mounted in glycerol. Hair cell losses, observed by phase contrast micro-

scopy, were counted and recorded in the form of cochleograms following the method of et al. (1974) and Coleman (1975). In each cochlear turn, cell losses in 3 rows of 100 outer cells (i.e. 300 in total) were noted and expressed as a percentage of cells in that row.

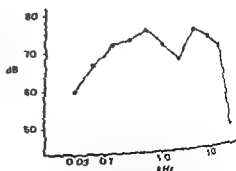
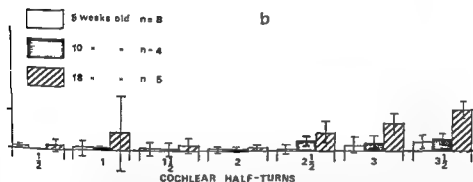
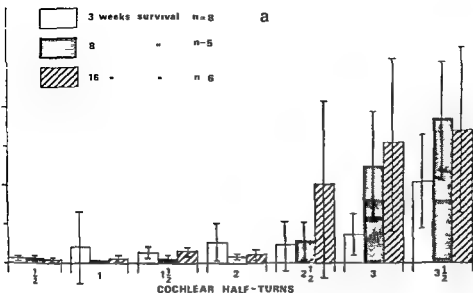


Fig. 1 The frequency spectrograph of the acoustic stimulus used in this study. ■ measured inside the box.



2 Histograms depicting the distribution of outer hair losses in experimental and control groups of guinea pigs. In (a) different regions of the cochlea are compared 8 and 16 weeks after acoustic exposure. In (b) control groups of ages corresponding to those of the three

experimental groups are shown. The histogram blocks represent mean values and the error bars represent standard deviations. n is the number of animals in each sample.

no inner hair cells were found to be missing, these were not included.

Control groups of similarly aged guinea pigs were kept alongside the experimental groups during the duration of the survival time to see if any hair cell losses resulted from natural ageing or uncontrolled environmental factors during this period. These control animals were killed at the same time as their experimental counterparts. The actual ages of all control animals were of course 2 weeks more than the experimental survival period, since they

were already one week old at the beginning of the experimental treatment which itself lasted for 1 week.

For statistical purposes each animal was taken as a single sample, the number of lost hair cells being the mean of those of the corresponding regions in the two cochleas (following Coleman, 1976a). Sample sizes are shown in Fig 2. As an examination of the hair cell losses in both the experimental and control animals showed that they followed an approximately normal frequency distribution,

Table II *An analysis of the ratio of outer hair cells lost in each of the three rows in turn 2 expressed as a percentage of the total outer hair cells lost in this region*

Outer hair cell row	Experimental survival time			Controls age 18 weeks
	3 weeks	8 weeks	16 weeks	
1	9.6 ± 17.0	3.3 ± 3.0	8.8 ± 12.2	10.7 ± 11.8
2	29.3 ± 38.1	35.2 ± 25.5	35.9 ± 25.2	23.2 ± 8.7
3	61.0 ± 38.2	61.4 ± 23.8	55.3 ± 35.4	66.1 ± 32.8

the Student's *t*-test was used to estimate the significance of hair cell deficits at different survival times and ages. In a number of cases the results of this statistical analysis were compared with those of a non parametric method (the Wilcoxon's rank sum or Mann-Whitney U-test) but no significant differences between the results of the two methods could be demonstrated.

RESULTS

The losses of outer hair cells in the spiral organs of 1-week old guinea pigs exposed to 7

days' continuous white noise and killed or 16 weeks later are shown in Table I and 2a. For comparison, hair cell losses in control groups are shown in Table I and 2b. Data for the 3 week experimental and control group are taken from our previous study (Douek et al., 1976).

It is evident that the total number of cells lost in the 8 weeks survival group is significantly higher than in the 3 weeks group, the further degeneration being particularly noticeable in half turn 3 and to a lesser extent in half turn 3½. However, between the 8 and 16 weeks' survival groups no further significant

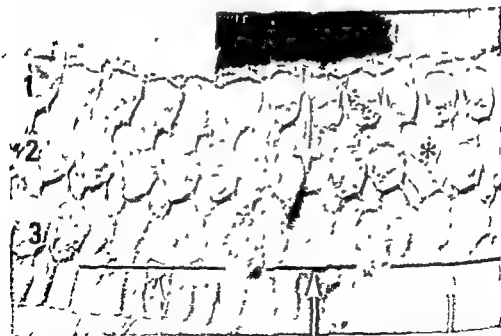


Fig 3 Micrograph of part of a surface preparation of half turn 3 eight weeks after exposure to 7 days' continuous white noise showing areas of damage in the three rows of outer hair cells (the numbers designate the rows). Note the presence of severe damage in row 3 moderate damage

including the recent formation of a phalangeal scar in row 2 and a single phalangeal scar in row 1. Missing cells are indicated by arrows and the recent scar by an asterisk. Interference-contrast microscopy.

ase is detectable except marginally in half, 2 and 1½ where the numbers lost are in case negligible

the control groups, no significant hair cell s were observed in animals aged 10 weeks responding to the experimental 8 weeks' (val group) in comparison with the 5-week-old animals, but in the 11 week old control group low but significant deficits had occurred in turn 3/3½

the majority of hair cell losses in experimental animals were in the outer two rows of outer hair cells following the pattern similar to that seen in natural ageing (Table II and 3)

DISCUSSION

These results confirm and extend our previous conclusion that continuous white noise in the 30 dB range is injurious to the cochleas of young guinea pigs (see Introduction). The absence of marked effects in the control groups shows that the losses in the experimental animals are due to exposure to this stimulus rather than to ageing or uncontrolled environmental noise, the notable increase in hair cell deficits in the cochlear turn 3/3½ between the 8 weeks' survival groups, where finally six of the outer hair cells had degenerated shows that the effect is quite prominent even at such low sound intensities

The distribution of damage along the spiral in the sound treated animals is similar to that seen in young guinea pigs exposed to prolonged periods of white noise at much higher intensities (about 120 dB) reported by Clark et al (1974) and it is reasonable to suppose that the type of trauma is essentially similar

The distribution of losses between the two rows of outer hair cells is also the same as that seen in other types of acoustic damage (Coleman, 1976). The threshold for acoustic damage therefore appears to be considerably lower in young animals than in adults in which 90 dB is usually taken as the

lower limit (Spoendlin 1976), and this finding emphasises the risk of exposing new born infants to constant noise even as low as 76 dB

These results also show that at least in young animals, 3 weeks is too short a period for the complete degeneration of affected cells and that 8 weeks' survival is a more satisfactory interval for this purpose. There appears to be no virtue in extending the period beyond this time, since no appreciable increase was seen at 16 weeks and at this time natural ageing processes begin to contribute significantly to hair cell losses, cell deficits seen in the 18 week old control animals are similar in size to those observed at about this age by Coleman (1976)

The reasons for the observed increase in hair cell deficits in the 3-8 week period are not clear from these experiments. Continued losses observed in this interval could be a consequence of variable rates of collapse and disappearance of cells killed by the initial stimulus, or perhaps of an increase in the susceptibility of mildly damaged cells to environmental noise. A third possibility is that some other component of the cochlea such as the stria vascularis, was injured by excessive acoustic exposure, leading to a general disturbance of cochlear homeostasis during this time

The restriction of damage to the apex of the cochlea is also interesting since it might be expected from the bandwidth of the white noise and its frequency distribution that damage would also be found in more basal parts of the cochlea. Coleman (1976) for example reported that acoustic over stimulation with a 4000 Hz tone at 119 dB caused maximum destruction in the region of the basal turn. We have recently observed by means of transmission and scanning electron microscopy that the cells of the apex in 1 week old guinea pigs show many signs of immaturity when compared with those of more basal regions (Dodson et al 1977) so that the distribution of damage may be related primarily to the developmental pattern of the cochlea rather than to its mechanical principles

ACKNOWLEDGEMENTS

We wish to thank the Medical Research Council for supporting this study financially. We are also grateful to Mr E. Cunnell for constructing the sound box, to Mr Keith Humphries for technical assistance, to Dr Rosemary Dalton for statistical advice, and to Dr Ade Pye for instruction in the surface preparation method.

ZUSAMMENFASSUNG

Junge Meerschweinchen wurden mit white noise (70–80 dB SPL) 7 Tage lang beschallt. Drei, 8 oder 16 Wochen nach der Beschallung wurden die Tiere getötet und die Cochlea wurde histologisch untersucht. Die Verluste von Haarzellen in den apikalen 3/3 Schneckenwindungen war deutlich erhöht nach 3 bis 8 Wochen Überlebenszeit, aber nicht nach 8 bis 16 Wochen. Hauptsächlich die äußeren zwei Reihen waren betroffen. Keine signifikanten Verluste wurden in den Kontrollgruppen (3–8 Wochen) beobachtet. In der Kontrollgruppe (16 Wochen) wurden kleine Verluste in der apikalen (3/3) Windung beobachtet, die auf Alterung zurückzuführen waren.

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A PHYSIOLOGICAL AND MORPHOLOGICAL STUDY OF THE COCHLEA OF THE RAT FOLLOWING TREATMENT WITH ATOXYL AND NEOMYCIN

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By means of physiological and morphological studies the inner ear pathology following exposure to toxic compounds atoxyl and neomycin was analysed in the rat. Primarily a high tone deterioration occurred with a subsequent morphological degeneration pattern among the hair cells in the basal part of the cochlea. Outer hair cells were more frequently affected than inner hair cells following the administration of both atoxyl and neomycin.

The normal and the pathological function of the labyrinth have been studied in a large number of animal experiments concerning the physiology and the morphology of the inner ear (Keidel & Neff, 1976, Smith & Vernon, 1976).

The guinea pig has been the most widely used experimental animal though also the chinchilla, the cat and others, and recently the lizard have also been studied (Sjöback & Wersäll, 1973, Bagger Sjöberg, 1977).

The evolution of inner ear changes following both ototoxic stimuli and aging, the cell degeneration pattern has been rather similar in the cochlea.

In the cochlea the earliest and most changes occur among the hair cells of the basal coils (McGee & Olszewski, 1962, Olszewski 1965, Elliott & McGee, 1965, Lund

quist & Wersäll 1966, 1967, Engström et al., 1966, Stebbins et al., 1969, 1973, Ylikoski, 1974). Thus Hawkins (1973) stated "that the hair cells of the cochlea react in a graded, sequential and stereotyped fashion to insults that exceed their tolerable limit, regardless of the immediate cause of injury".

The atoxyl induced sequence of hair cell damage appears to be in contrast to the general pattern and can be selectively limited to the apical part of the guinea pig cochlea (Anniko, 1976). The mechanism of this effect is, however, not known.

Lesions restricted to the apical coils so far have been reported only following experimental obstruction at various levels of labyrinthine blood vessels (Kimura & Perlman 1958, Bernstein & Silverstein, 1966, Alford et al., 1965) and in cases of idiopathic labyrinthine hydrops (Kohut & Lindsay, 1972). However, the pathological changes in the inner ear of such lesions were not restricted to the hair cells only but involved also other structures of the membranous labyrinth.

The aim of the present investigation was to study whether or not the atoxyl induced location of hair cell damage is specific to the guinea pig or if this pattern can be reproduced also in other species, e.g. the rat as used in

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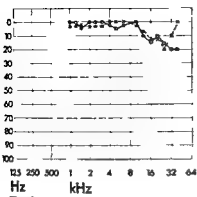
ZUSAMMENFASSUNG

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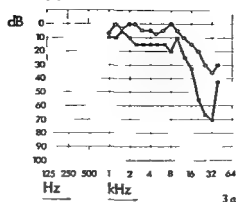
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A23

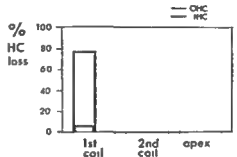
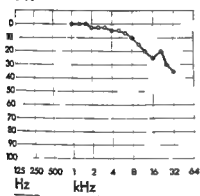


A19



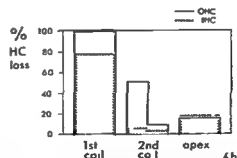
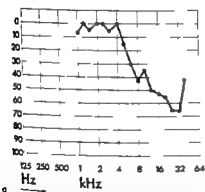
3a

A7



3b

N11



4b

Fig. 1 7 9 11-12 N₁ N₂ threshold shift curves. The divergence measured in dB in the experimental animals of the threshold shift relative to the normal threshold was registered as hearing impairment (vertical axis). The investigated frequencies were marked on the horizontal axis. A atoxyl N neomycin Fig. 1 Animal no. A23
 Fig. 2 N₁ N₂ threshold shift curve Animal no. A7
 Fig. 3 Animal no. A19 (A) N₁ N₂ threshold shift curve

■—■ left ear ○—○ right ear Differences occur in threshold shift between inner ears of the two sides (B) Histogram illustrating cochlear hair cell loss of the left ear. The sensory cell damage was primarily confined to the outer hair cells of the basal half of the 1st coil.
 Fig. 4 Animal no. N11 (A) N₁ N₂ threshold shift curve ●—● right ear (B) Histogram illustrating the extent of hair cell damage of the right ear ■ various coils of the rat cochlea

Morphological preparation technique

Following decapitation of the animal the inner ear was explored via the bulla tympanica after removal of the auditory ossicles. The stapes was extracted, the round window membrane removed and a hole was made at apex and to the lateral (horizontal) semicircular canal. The labyrinth was locally perfused with a 2% osmic acid solution in Veronal acetate buffer (pH 7.2–7.4), a technique originally described by Wersäll (1956) concerning the guinea-pig.

The specimens remained in the fixative for 2 hours and were thereafter treated according to standard methods for biological electron microscopy (Hayat, 1970) and embedded in Epon (Luft, 1961). Embedded cochleae were divided along the mid modiolus, sectioned and mounted for mapping of the cochlear hair cells, according to the surface preparation technique for cytochromeograms (Ernstsson, 1972; Anniko, 1976). Representative areas from the surface preparations were investigated by light microscopy (staining toluidine blue) and electron microscopy (Anniko & Wersäll, 1975).

RESULTS

Individual differences occurred among animals in susceptibility to atoxyl and neomycin with regard to N_1 , N_2 threshold shift as well as to morphological change. The animals were in good clinical condition at the time of sacrifice. The animals treated with atoxyl showed a normal weight increase during the treatment period, while those treated with neomycin showed a less than normal weight gain during that period. The animals were grouped with regard to the slope of the N_1 , N_2 threshold shift curve in three subgroups independent of the drugs administered and the dose.

Minimal threshold shift

This group comprises animals with a 10–30 dB threshold shift over one or two octaves in the frequency range studied (1–40 kHz). The animals receiving 50 mg/kg of atoxyl all belonged to this group (10 rats) together with one rat

treated with 75 mg/kg of atoxyl (total 350–650 mg/kg vis à vis 375 mg/kg). Threshold shift occurred without exception in the octave bands 8–32 kHz (Fig. 1) and similar in both ears.

The difference in threshold shift between animals receiving the same total amount of atoxyl was only minimal when investigated 45 or 62 days following the last administration.

The morphological analysis of surface preparations revealed a uniform outer hair cell (OHC) damage pattern among the animals belonging to this group which showed only a minimal (1–4.8%) loss of OHCs evenly distributed in the basal coil. Both outer and the inner hair cells (IHC) of the apical region and the upper part of the 2nd coil appeared intact. In general the histological sections showed a normal structure. They were intact in all but one of the investigated cochleae (A7).

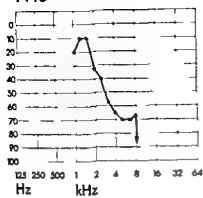
One animal (A7), however, showed almost 80% degeneration of OHC in the part of the 1st coil, though most of the cells were well preserved (Fig. 2). In contrast, the upper half of the basal coil showed a limited hair cell damage—less than a few percent. There was only a small threshold shift amounting to 35 dB at 31.5 kHz, while at other frequencies were considerably affected (10 kHz, 15 dB; 12 kHz, 20 dB; 14 kHz, 25 dB; 20 kHz, 20 dB; and 25 kHz, 30 dB). Two animals receiving 50 mg/kg of atoxyl on each occasion (totally 350–400 mg/kg) showed threshold shift and their cytochromeograms showed loss of only single OHC in the basal coil in no part exceeding 1–3%.

None of the neomycin treated animals belonged to this group with minimal change in the hearing threshold.

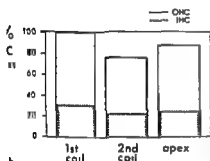
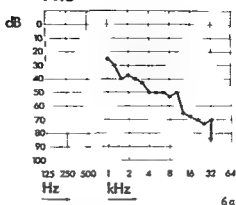
Sloping threshold shift

This classification was used when the threshold shift was at least 30 dB in a frequency range of 1 or 1½ octaves. Both atoxyl and neomycin treated animals revealed this type of hearing impairment.

N16



A10



N15

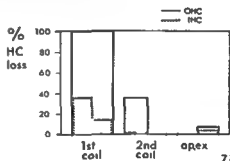
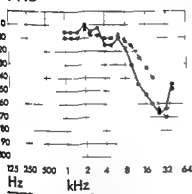


Fig 5 N₁ N₁ threshold shift curve. Animal no N16 right ear. No electro-physiological response was obtained concerning frequencies above 8 kHz.

Fig 6 Animal no A10 Right ear (A) N₁ N₁ threshold shift curve. ●—● right ear (B) Histogram illustrating severe degeneration of outer hair cells at all levels of the cochlea while many inner hair cells still were identified in the surface preparations (C) Light microscopy (LM). Degeneration of outer and inner hair cells. The structural organization with an inner and an outer tunnel is still preserved.

Fig 7 Animal no N15 (A) N₁ N₁ threshold shift curve. ●—● right ear ■—■ left ear (B) Histogram of the right ear illustrating severe degeneration of outer hair cells in the basal part of the cochlea. The inner hair cells appear considerably more preserved even in the basal coil (C) Interference contrast light microscopy. Surface view from the basal part of the second coil showing damage to outer hair cells in all three rows (OHC I-III) while the inner hair cells (IHC) all are preserved at this distance.

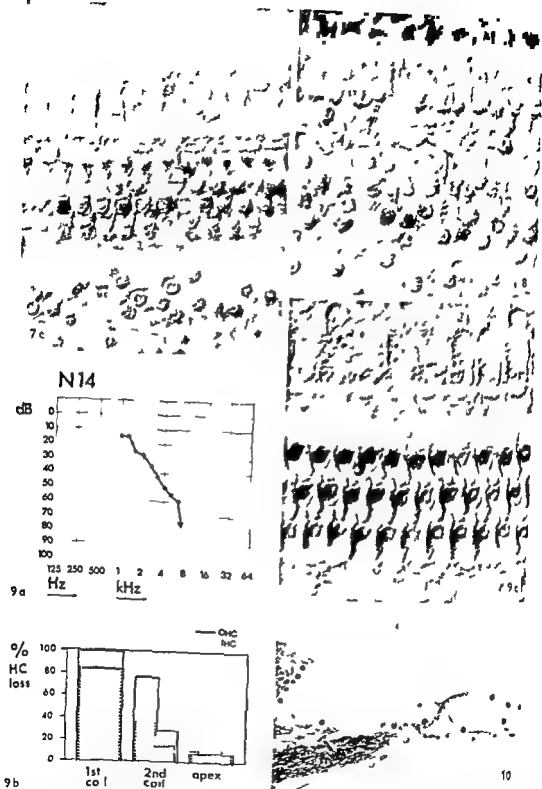


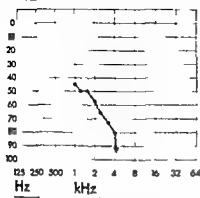
Fig. 8 Interference contrast light microscopy. Animal no. N11. Basal coil. Total degeneration of outer and inner hair cells.

Fig. 9 Animal no. N14. Right ear. (A) N₂ threshold shift curve. (B) Right ear. An impairment of hearing was found concerning the high frequencies. No detectable hearing could be recorded in frequencies above 6.3 kHz. (C) Histogram illustrating severe damage to the hair cells

of the organ of Corti in the basal and middle parts of cochlea. (C) Interference contrast light microscopy. Animal outer (OHC I-III) and inner (IHC) hair cells in apical half of the second coil.

Fig. 10 LM. Animal no. N24. 2nd coil. All hair cells degenerated and the structural differentiation of supporting cells is lost.

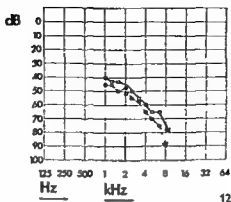
A22



11 N_1 N_2 threshold shift curve Animal no A22
right ear Severe hearing loss

12 N_1 N_2 threshold shift curve Animal no N24

N 24



●—● right ear □—□ left ear The degree of hearing impairment is roughly the same in the inner ear on both sides

This group consisted of totally 14 animals, all of which belonged to the atoxyl—(75 mg/kg bw on each occasion except one rat—10—which received 125 mg/kg/day three times) vis a vis neomycin treated (100 mg/kg on each occasion) rats. The atoxyl induced threshold shift mainly involved frequencies above 8 kHz (Fig. 3) while the neomycin damage could affect all frequencies above 2 kHz (Figs 4, 5).

A steep sloping threshold curve was observed in several animals (A 19: 10 kHz, 10 dB, 2 kHz, 25 dB, 16 kHz, 32.5 dB, 20 kHz, 5 dB, 25 kHz, 70 dB, 31.5 kHz, 70 dB and 40 kHz, 42.5 dB). Some atoxyl treated animals in this group displayed differing degrees of threshold shift in both ears of the same animal (Fig. 3).

The histopathological examination revealed in one animal (Fig. 6C) with a more extensive hearing loss (A 10: 1 kHz, 25 dB, 2 kHz, 37.5 dB, 4 kHz, 50 dB, 8 kHz, 52.5 dB, 16 kHz, 75 dB, 31.5 kHz, 72.5 dB) severely damaged in the whole cochlea (78–90%) while the IHC were considerably less affected (20–30%). The three rows showed equal OHC

The neomycin treated animals showed an initial threshold shift as a high tone loss with a threshold elevation of 10–20 dB at

the lower frequencies. There appeared a good correlation between the threshold of the two ears of the same animal. Two animals displayed no threshold shift at frequencies 1–8 kHz, whereafter the threshold decreased rapidly e.g. (N 15: 8 kHz, 15 dB, 10 kHz, 30 dB, 12 kHz, 45 dB, 16 kHz, 55 dB, 20 kHz, 60 dB, 25 kHz, 70 dB, 32 kHz, 65 dB, and 40 kHz, 45 dB) (Fig. 7). The histopathological examination revealed extensive damage in the basal coil of the OHC which were totally lacking. The IHC were considerably better preserved, showing a 30–40% loss in the basal part and less than 20% were degenerated in the apical part of the basal coil. Unfortunately it was not possible to investigate the basal hook in these animals. In the basal part of the 2nd coil 36% of the OHC and 3% of the IHC had suffered damage (Fig. 7C) while the upper part of the coil showed a normal hair cell count. This was also true concerning the apical coil except for the most apical mm of the cochlea which revealed irregularities among both OHC and IHC.

With increasing threshold shift (Fig. 4) in the range between 4 and 8 kHz (N 11: 5 kHz, 15 dB, 6.3 kHz, 30 dB, 8 kHz, 45 dB) there was an increased hair cell damage as revealed by microscopic examination. In the basal coil apically 80% IHC were missing and the

OHC had totally disappeared (Fig. 8). In the basal part of the 2nd coil 55% of the OHC were missing and 12% of the IHC but the upper part of the same coil still appeared mainly intact. The apical coil demonstrated 20% missing IHC and somewhat fewer OHC. The hearing in frequencies 1–4 kHz was normal.

Comparison of the threshold shift with the light microscopic interference contrast findings revealed in general a close correlation between the hair cell degeneration pattern and the threshold shift. Animal N14 (Fig. 9A), showed an evenly descending threshold shift curve from 1 kHz to 6.3 kHz (1 kHz, 15 dB, 1.6 kHz, 25 dB, 2 kHz, 30 dB, 3.1 kHz, 40 dB, 4 kHz, 50 dB, 5 kHz, 55 dB, and 6.3 kHz, 60 dB). Above that frequency no measurable response was recorded. The histological examination disclosed that well preserved hair cells occurred only in the upper part of the 2nd coil (Fig. 9B). At apex both types of hair cells showed irregularities, though less than 10% were actually missing.

Form threshold shift

Animals recorded in this group had threshold shifts of 30–40 dB and often combinations with no physiological response within a part of the investigated frequencies. This type of inner ear damage was found both in animals treated with atoxyl and neomycin (100 mg/kg b.w. on each occasion of both substances respectively with a total dose of 1200 mg/kg of neomycin and 400 mg/kg of atoxyl). The threshold shift was rather similar in both groups, often showing an impairment of 40–50 dB at 1–8 kHz (Figs. 11, 12).

The time between the last injection and the sacrifice of the animal was of great importance following neomycin administration. In animals having received the same total amount of neomycin and investigated 45 days following the last injection only 1 of 6 rats had a total threshold shift at frequencies above 1 kHz compared with 5 of 6 in the group measured 102 days after the end of the treatment.

The histological examination of the cochlea

showed severe damage to the OHC which often were present only in the most apical of the cochlea. The IHC were also severely affected in many parts of the cochlea. Scattered smaller groups of both OHC and IHC could be found at several levels in the cochlea though not in the basal coil. In a profusely destroyed cochlea also the supporting structures revealed significant damage in many places, especially in the basal coil. The entire structural organization of the organ of Corti had disappeared (Fig. 10).

Deaf animals

Two animals were injected with 200 µl/day of neomycin with a total dose of 140 kg. No response at all could be elicited investigated 7 respectively 26 days following the last injection.

DISCUSSION

A variety of ototraumatic agents are known and their effects on hearing are well documented (review Hawkins, 1976). In the field of comparative otopathology Hawkins (1976) described how noise, drugs and ageing could induce indistinguishable patterns of cochlear pathology with subsequent hearing impairment or even total loss.

In the present investigation the first physiological threshold changes in the response appeared in the high frequency range 20–4 kHz following both atoxyl and neomycin administration thus indicating a hair cell lesion in the basal part of the cochlea. This is in agreement with the results of the morphological investigation where hair cell loss in various degrees were found in animals in which the threshold shift was considerable. Initially, however, only a scattered hair cell degeneration was observed in the basal coil without any predominance with regard to OHC and IHC.

In animals that displayed a minimal threshold shift without distinct hair cell degeneration in a specific region of the cochlea this

interpreted in two ways either the threshold shift is a result of a dysfunction of only the apical metabolism of some hair cells while the basal metabolism still continues sufficiently or it is a result of an imbalance of the whole hair cell homeostasis though without being fully degenerated. Since the threshold measurement and the histopathological findings of the present investigation were made after the last administration of the drugs, the latter hypothesis seems the least probable reversible effect (disturbance of the functional metabolism) on hair cells following neomycin administration has been reported by Wersall & Flock (1964). Morphological and physiological correlates on hair cells with impaired metabolism have been reported by Karickhoff et al (1976).

The location of the basilar membrane of atoxyl induced hair cell degeneration in the cochlea thus appears to be species dependant. Anniko (1976) showed apical affection of OHC and IHC as a result of atoxyl administration in the guinea pig while in the present study no selective damage occurred among hair cells in the apical coil of the rat cochlea. On the contrary the earliest and most frequent hair cell changes were found in the basal part of the rat cochlea.

In comparison, the neomycin induced hair cell changes occur mainly in the basal coil both in the guinea pig (Stebbins et al, 1969, Jauhainen et al, 1972, Ylikoski, 1974) and the rat, the latter confirmed by the present study.

In atoxyl treated animals with a sloping threshold shift discrepancies in the threshold shift sometimes appeared in both ears of the same animal while the inner ear pathology of neomycin treated animals revealed a rather uniform picture in the two sides. The reason for this pattern may be explained by a mechanism for a semipermeable system controlling the blood/endolymph barrier. Stupp (1970) showed that the serum level of aminoglycoside antibiotics must exceed a certain level before an inflow to the endolymph occurs. Similar results were obtained by Ritter et

al (1971). It is thus likely that the neomycin concentrations in the blood following the injections were sufficient during a period of time to allow passage through such a barrier while an administration of 75 mg/kg of atoxyl was very close to the borderline level for inflow to the cochlea. Variations among individual animals in kidney function and circulation of inner ear fluids may also influence the susceptibility to ototoxic substances.

Following a penetration into the labyrinth, both atoxyl and neomycin are retained in the cochlea during a long time and have a delayed elimination from the inner ear (Stupp, 1970, Stupp et al, 1973, Anniko & Plantin, 1977). However, both atoxyl and neomycin are nephrotoxic (Anniko & Ljungquist, 1977, Hawkins, 1970) which following a prolonged administration may cause an accumulation of the substances in the body and thereby exert ototoxic effects in the concentrations initially not affecting the hair cells of the labyrinth.

No significant difference occurred among atoxyl treated animals receiving approximately the same total amount when investigated 18 days to 3 months after the last injection in contrast to the neomycin induced hair cell degeneration, which during the first 1-2 months progressed with the survival time. Several investigators have focused attention on such a mechanism in the development and course of hair cell damage following the administration of aminoglycoside antibiotics (Ylikoski, 1974, Anniko & Wersall, 1977, unpublished observations).

A correlation between the pure tone audiogram and the cochlear pathology has been well documented in the guinea pig (Ylikoski, 1974), the monkey (Stebbins et al, 1969), the cat (Schuknecht & Sutton, 1953, Elliott & McGee, 1965), in man (Bredberg 1968) and others, while the rat, often used in physiological experiments, lacks comparative documentation.

The number of animals with an abrupt hearing loss showing a relatively distinct change in hearing sensitivity at a narrow frequency range in the present study, however, was too

small to allow a detailed analysis of the anatomical frequency scale in the rat. Roughly, this may be interpreted as follows: the lower half of the basal coil has an area of maximal stimulation at frequencies above 20 kHz, the upper half of the basal coil corresponds to 10–20 kHz, the lower half of the 2nd coil includes 2–8 kHz and the upper half of the same coil contains frequencies of maximal stimulation below 1.5–2 kHz. The importance of the apical region of the cochlea in hearing is, however, still not established.

Some biologically active substances may have a high order of organ specificity but this does not necessarily mean that the location of their greatest effect within the organ is similar in different species. In a complex system such as the inner ear species-dependant variations can occur, e.g. as shown in the present study concerning atoxyl and neomycin. Experimental pathology may benefit from studies on the differences in the pharmacological action(s) on the inner ear in different species of various ototoxic substances and the result of such studies may result in bringing insight into the function of the ear.

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ZUSAMMENFASSUNG

Die Pathologie des Innenohres zufolge der ototoxischen Verbindungen Atoxyl und Neomycin wurde in der Ratte mittels physiologischer und morphologischer Methoden analysiert. Trotz der Verschiedenheiten in ihrer Wirkungsart verursachten beide Substanzen in den Anfangsstadien ihrer ototoxischen Wirksamkeit in der Ratte eine Deterioration im Hören von höheren Tönen mit einem folgenden morphologischen Degenerationsmuster in den Haarzellen in den Basenteile der Cochlea. Die äusseren Haarzellen waren bedeutend öfter angegriffen als die inneren Haarzellen zufolge der Anwendung von sowohl Atoxyl wie Neomycin.

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A PROSPECTIVE STUDY OF GENTAMICIN OTOTOXICITY

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Abstract Twenty patients were included in a prospective otoneurological study performed to assess the ototoxicity in gentamicin therapy. Gentamicin was administered intravenously, and the serum level was currently determined. Audiographic and electronystagmographic studies were carried out at the institution and discontinuation of the treatment and again a few weeks later. Ten patients exhibited ototoxic actions, predominantly cochlear, 4 of the cases being fully reversible. Two patients developed severe hearing loss, associated in one with bilateral extinction of vestibular function. Low serum levels of gentamicin did not rule out the possibility of ototoxicity. These results urge the continuing of prospective studies and indicate that gentamicin should be used only as a link in the primary treatment of severe infection or in cases in which other, less toxic agents have failed.

Side effects of drugs upon the 8th cranial nerve are generally due to salicylates, certain diuretics, and aminoglycosides. Among the latter a few affect mainly the vestibular and others especially the cochlear system.

In retrospective studies the ototoxicity of gentamicin has been found to disturb vestibular function in two thirds of the cases and cochlear function in one-third. Half the patients with signs of hearing loss also exhibit vestibular symptoms (Jackson & Arcieri, 1971).

In animal experiments using electron microscopy Wersall et al (1969) demonstrated that the ototoxic action of gentamicin consists in degeneration of the vestibular and cochlear ciliated cells. The damage to the ciliated cells in the cochlear is less marked and manifest later than the vestibular changes. In the organ of Corti, degeneration of the ciliated cells starts in the basal turn, that of high frequency perception, and progresses towards the apex.

The damage to the sensory cells is possibly

dependent on the concentration of gentamicin in the endolymph. Hawkins (1973) has emphasized, moreover, that the ototoxic might be due to changes in the glucocatabolism of the ciliated cells or to change the composition of perilymph and endolymph caused by the vascular changes even gentamicin.

It still remains unelucidated whether a high concentration in the blood at a certain time after parenteral administration of gentamicin (the peak concentration) is a factor in the ototoxicity, or whether it is rather a variation of an elevated concentration induced by the gentamicin level in the blood immediately before the next dose (the valley concentration) which is decisive (Banck et al, 1974; Hewitt, 1974; Noone et al, 1974; Nonne et al, 1973). Possibly both factors are operative, as it may be the area of the 24-hour concentration curve which ought to be relevant. Possible signs of ototoxicity (Lancet Tjernstrom et al, 1973).

The incidence of gentamicin induced toxic damage has been inadequately evaluated, as the publications have so far been based upon retrospective studies.

The present investigation was an attempt to assess by a prospective study the incidence of toxic damage by gentamicin to the 8th cranial nerve.

PRESENT INVESTIGATIONS

Material and Method

The material comprises 20 patients (12 males and 8 females) admitted to Medical Def

entofte Hospital from 1 11 73 to 28 2 75
 these patients 17 were suffering from a
 nant, radical haematological disease (in
 ng 8 with acute leukaemia) and three from
 minated collagen disease. None have
 on previous treatment with gentamicin.
 one had prior otologic diseases. The in
 ion for gentamicin therapy was severe in
 n which had usually arisen during inten
 cytostatic combination therapy. During
 above mentioned period all patients were
 ided in the material, if their condition per
 d the fairly exacting examinations for a
 ile of hours in the E N T Department
 in the first 48 hours of the antibiotic treat
 t. The mean age was 49 years (range 16-
 years). Gentamicin was often combined
 cephalothin and administered intrave
 sly in the course of 5 minutes. The initial
 * was calculated on the basis of body
 ght (80 mg for patients over 60 kg, 60 mg
 those under 60 kg), while the subsequent
 and the intervals were adapted also to
 serum creatinine. Thereafter, the dose was
 d on the basis of the serum concentration
 gentamicin which was measured for the first
 e on the 2nd-3rd day of the medication.
 * serum concentration of gentamicin was
 ermined by an Agar well diffusion test. De
 mination of the concentration as well as of
 -m creatinine were repeated at intervals of
 days, done on samples drawn immediate
 before an injection (valley concentration)
 45-60 minutes later (peak concentration).
 aim was a peak concentration of 6-12 $\mu\text{g/l}$
 and/or a valley concentration of 1-3 $\mu\text{g/ml}$.
 he mean duration of treatment was 8 days
 16 days) and the total gentamicin dose
 d from 480 mg to 3260 mg (mean
 3 mg).

In the event of normal otoscopy the patients
 the following tests before or in the course
 the first 2 days of the treatment:

- pure tone audiometry and speech audiometric hearing test (T_1),
- vestibular testing by electronystagmography to detect spontaneous and posi

tion nystagmus as well as a caloric test
 stimulating by 44°C water for 30 sec by
 the method of Hinchcliffe (ENG) (2, 7).

These tests were repeated a few days after
 discontinuation of the medication (1st follow
 up) and again 3-19 weeks (mean 10 weeks)
 later (2nd follow up).

Four patients died between 1st and 2nd fol
 low up.

RESULTS

(a) Audiological

To assess cochlear function and to exclude the
 test-retest variation (Harris & Myers, 1954),
 we have defined the hearing loss at the individ
 ual frequencies as a reduction of the hearing
 threshold by more than 10 dB relative to the
 initial value. The results are seen in Tables I
 and II.

As might be expected, all hearing losses
 were most pronounced for the high frequen
 cies.

At 1st follow up, unilateral hearing loss was
 found in 3 out of 20 patients. In one the hearing
 loss was present also at the 2nd follow up ex
 amination. In another it was versible, while
 the third patient had unchanged hearing loss at
 2nd follow up. In the meantime, however, this
 latter patient had been put on streptomycin,
 and there was also a suspicion of leukaemia
 involving the central nervous system, so that
 the result of the 2nd follow up cannot be taken
 into account. No patient developed changes
 in T_1 .

At the 2nd follow up 6 of 16 patients were
 found to have unilateral and one patient bilat
 eral hearing loss in the range 15-30 dB (mean
 20 dB). The serum gentamicin had been higher
 than intended in only 2 of these 7 patients. In
 the only 2 patients who could be tested again
 one year later the hearing impairment proved
 to have been fully reversible. Two of the 7 pa
 tients also developed a change in T_1 of more
 than 10 dB, both had too high serum genta
 micin. It should be mentioned also that, before
 the 2nd follow up, 2 of the patients had re

Table I *Distribution of hearing loss in 3 patients at 1st follow-up and in 7 patients at 2nd follow up (one patient had a hearing loss at 2000 Hz as well as 4000 Hz at 1st follow up)*

Ti Threshold of intensity (speech reception threshold)

No of pats tested		Frequency (Hz)			
		2000	4000	8000	Ti
20	1st follow up	1	1	2	0
16	2nd follow up	1	1	5	2

ceived 2 and 3 courses respectively of gentamicin at intervals of a couple of weeks (total treatment period 11 and 16 days and total dose gentamicin 2 640 and 3 040 mg respectively)

(b) Vestibular

At 1st follow up 2 of the 20 tested patients had affected vestibular function. One of them had bilateral canal paralysis which proved to be permanent. This patient had had a massive elevation of the valley concentration of gentamicin in the serum (5.6 µg/ml). The other patient had unilateral canal paresis which regressed in 8 weeks. In this case the gentamicin concentration had been within the intended range.

At 2nd follow up, performed in 16 cases, bilateral canal paralysis was still present in the above mentioned patient. Another patient had unilateral canal paresis which proved to have been reversible at repeated follow up one year

later. The serum gentamicin in this patient had been within the intended range.

DISCUSSION

So far, the real incidence of gentamicin-induced ototoxic damage has been unknown. In the analyses published hitherto have been retrospective. This applies to the studies of Banck et al (1973), Noone et al (1974), Västström et al (1973) and Tjernström et al (1973) who demonstrated by acoustic reflex tests, ototoxicity—as a rule sub-clinical (asymptomatic)—in 20% of the treated patients. Ototoxicity causing symptoms was recorded by Jackson & Arcieri (1971) in one of a very large patient material in the USA during the period 1966–69. Due to more careful monitoring of the treatment, this rate seems to have fallen since then to around 1% (Harris 1974). A similar rate of ototoxicity with symptoms has been reported by Federspil & Burg (1970). Since the hearing loss is usually primarily the highest frequencies, it is not noticed by the patient who is also not corrected by reflex for mild canal paresis of the vestibular apparatus. This explains the low rate of ototoxicity in the last mentioned series.

The present prospective study demonstrated a very high degree of apparently gentamicin-induced ototoxicity. Almost half the patients (9/20) developed hearing loss. In

Table II *Ototoxicity probably related to therapy with gentamicin in relation to age and valley concentration (HVC) and the serum creatinine*

Symptom	No of pats	Mean age (year)	Mean HVC (µg/ml)	Serum creatinine mean (mg/dl)
Auditory only				
Transient	2	52 (36–68)	2.4 (1.6–3.2)	0.8 (0.7–0.9)
Irreversible	5*	38 (17–55)	1.8 (1.0–3.2)	1.0 (0.5–1.5)
Vestibular only				
Transient	1	79	2.4	1.6
Irreversible	0			
Auditory and vestibular				
Transient	1	17	1.0	0.7
Irreversible	1	75	5.6	1.0

* One patient also received streptomycin

ages were fully reversible, the other 6 died relatively short time after the second follow-up. In at least 2 of the patients the hearing impairment was appreciable, being demonstrable by conventional as well as by speech audiometry.

Except in one patient, with coming bilateral extinction of vestibular function the hearing damage was unilateral. Three of 20 patients tested exhibited vestibular dysfunction which was unilateral and fully reversible in two. Two patients developed signs of hearing and vestibular damage, fully reversible in one.

Thus at worst, there is the possibility of permanent ototoxicity (as a rule sub-clinical) in 6 of 20 patients. In most cases the toxicity was not demonstrable at the completion of treatment, not until the 2nd follow-up examination some weeks later. The cause of this incidence of hearing damage is possibly the poor general condition of some of the patients who, therefore, did not cooperate very well.

Judging by the case notes, however, the general condition—and the haemoglobin level—did not seem poorer at the subsequent examinations than at institution of the treatment. None of the patients showing ototoxicity was on furosemide therapy, and only 2 of the patients with ototoxicity developed a slightly elevated serum creatinine in the course of the treatment, this might have given rise to non recorded major fluctuations in the gentamicin concentration. Two of the patients with ototoxicity were over 70 years of age, the mean age in this group was 46 years, as compared with 49 years in the group without ototoxicity. Only one of the patients complained spontaneously of hearing impairment (and dizziness). This is in agreement with the studies reported above in which 1–2% of the patients developed subjective symptoms of ototoxicity.

It has been pointed out by Hewitt (1974) that the valley concentration of gentamicin in the serum is particularly of value in preventing toxicity. A valley concentration exceeding 1.5–2.0 $\mu\text{g/ml}$ is said to involve an in-

creased risk of damage (Banck et al, 1973, Hewitt, 1974, Nordstrom et al, 1973).

Among the present 10 patients with ototoxicity, 4 had valley concentrations exceeding 2 $\mu\text{g/ml}$ (2.4, 3.2, 3.2, and 5.1 $\mu\text{g/ml}$ respectively). The highest valley concentration (5.6 $\mu\text{g/ml}$) was observed in the patient with bilateral canal paresis and severe bilateral hearing loss. Only one patient had an elevated peak concentration of gentamicin in the serum (13.5 $\mu\text{g/ml}$)—and incidentally also a slightly elevated valley concentration (3.2 $\mu\text{g/ml}$). This patient developed severe unilateral hearing loss after having been treated, with minor interruptions, for 16 days with gentamicin in a total dose of 3040 mg. In the last 2 patients with a slightly raised valley concentration the ototoxicity proved fully reversible. It must be mentioned that in 4 patients without demonstrable ototoxicity the valley concentration had also exceeded 2 $\mu\text{g/ml}$ (2.5, 3.6, 3.6, and 6.6 $\mu\text{g/ml}$), but only 2 of these patients were seen at the second follow-up. In other words, even a very low valley concentration (<2 $\mu\text{g/ml}$) does not exclude the possibility of ototoxicity. Similarly, valley concentrations up to at least 3.6 $\mu\text{g/ml}$ have been observed without subsequent otological damage. There does not seem to be any question of major fluctuations in the serum level of gentamicin, leading to difficulty in regulating the dosage in the patients with ototoxicity in contradistinction to those without. At least, there was no difference between the two groups in the frequency of dose adjustments.

As regards the total dose of gentamicin and the duration of treatment, our material shows no difference between the group of patients with and that without demonstrable ototoxicity. Thus, 10 patients with ototoxicity received a mean dose of 1559 mg gentamicin (480–3040 mg), and the treatment period had averaged 8.0 days (5–16 days), whereas the 10 patients without demonstrable ototoxicity received an average of 1762 mg gentamicin (960–3260 mg) in the course of a mean treatment period of 7.9 days (5–12 days).

CONCLUSION

The present material is fairly small and nearly all the patients had malignant haematological disease treated intensively with cytostatics prior to the infection. Owing to our favourable experience of controlling infection with gentamicin, it was not justified to run a control group of similar patients from whom gentamicin was withheld.

Since ototoxicity proved to be a more common complication of gentamicin therapy than was earlier assumed, our results prompt us to continue with prospective studies, including patients with other radical diseases and including also other aminoglycosides for toxicological comparison.

As a rule, the demonstrated toxicity does not give rise to symptoms. It generally affects the cochlea, is often unilateral and presumably fully reversible in most cases. Ototoxicity was observed in several patients with a very low serum level of gentamicin (valley concentrations of less than $2 \mu\text{g/ml}$). Accordingly, the value of determining the serum level lies more in securing optimal treatment than in excluding a risk of ototoxicity.

Gentamicin should still be used only as a link in treating severe infections and infections in which less toxic agents have proved ineffective. The treatment should be as brief as possible.

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ZUSAMMENFASSUNG

In einer prospektiven otoneurologischen Untersuchung, welche 20 Patienten umfaßte, wurde versucht, die Ototoxizität von Gentamycin zu beurteilen. Gentamycin wurde intravenös appliziert und dessen Serumkonzentration laufend bestimmt. Bei Beginn unmittelbar nach Abschluß der Behandlung sowie einige Wochen später wurde ein Audiogramm aufgenommen sowie Elektronystagmographie durchgeführt. Bei 10 Patienten konnten ototoxische Schäden festgestellt werden, welche überwiegend

cochlear und in mindestens 4 Fällen reversibel waren. Zwei Patienten zogen sich Horschaden zu, in einem der Fälle sogar mit Vestibularfunktion. Selbst bei niedriger Gentamycin-Konzentration (Talspiegeln unter $2 \mu\text{g/ml}$) wurde Ototoxizität beobachtet. Es wird darauf hingewiesen, daß Gentamycin nur als ein Teil der Behandlung schwerster Infektionen vorbehalten sei, oder wo andere, weniger toxische Medikamente wirkungslos erwiesen haben.

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FLUORESCENT STAINING OF HAIR CELLS WITH ETHIDIUM BROMIDE

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Abstract The present study evaluated ethidium bromide, a nucleic acid specific fluorescing stain for cochlear applications. Tissue exposed to acoustic stimulation did not show the loss of fluorescence in hair cells described in other fluorescing stains. The ethidium bromide fluorescence technique was, however, found to be useful in detecting subtle damage in cell nuclei even before gross structural alterations in cochlear cytoarchitecture appeared. The implications of the use of ethidium bromide staining for histologists are discussed.

Changes in fluorescence in hair cells following acoustic stimulation were reported by Goldstein (1973) and Guth et al. (1974). Goldstein (1973) using the appearance of the fluorescent end product (fluorescein) of the enzymatic hydrolysis of the non fluorescent fluorescein diacetate (FDA) as an indicator of enzymatic activity in living cochlear tissue, observed progressively diminished fluorescence (metachromasia) induced by varying degrees of acoustic stimulation. Goldstein concluded that since the rate of hydrolysis of the FDA to fluorescein did not change after acoustic stimulation, the loss of fluorescence was due to changes in cell membrane permeability. Guth et al. (1974) investigated the effect of the organ of Corti by terphenyl-3-methylolcholinium 3 (TPHC-3), a fluorescent derivative of hemicholinium 3, an inhibitor of acetylcholine synthesis (MacIntosh et al., 1958). Guth and his associates intended to use TPHC-3 to map the efferent (cholinergic) olivo-cochlear bundle (OCB) innervation of the organ of Corti. The results of their study on fixed cochlear tissue, however,

suggested that TPHC-3 was not taken up by efferent nerve endings but by what the authors surmised to be cell nuclei. After exposing cochlear tissue to ototoxic or acoustic trauma, Guth et al. observed a loss of fluorescence associated with hair cell nuclei. Because it was felt that the TPHC-3 cation might be reacting with acidic moieties in cell nuclei such as nucleic acids, it was decided to use ethidium bromide (ETHBr), a nucleic acid specific fluorescing stain (LePecq et al., 1964, LePecq & Paoletti, 1967).

The present study was designed to evaluate ETHBr for cochlear applications. Animals were subjected to acoustic trauma to determine whether ETHBr exhibited a stimulation-dependent reduction of fluorescence as described by Goldstein (1973) and Guth et al. (1974). Under the conditions used in the present experiment, no loss of fluorescence was observed in cochlear tissue after sound exposure but other valuable observations made in the course of this study are reported here.

METHODS

Guinea pigs weighing between 200 and 500 grams with active Preyer reflexes were used. Animals were sacrificed by decapitation and prepared for histological study using the following procedure. Temporal bones were im-

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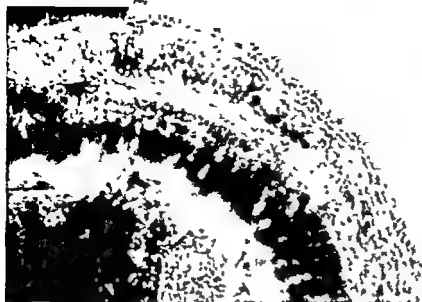


Fig 1 UV photo showing fluorescent pattern produced by ETHBr fixation. Magnification $\times 100$

mediately removed and their bullae opened. Tiny openings were made in the apical and basal ends of the cochleae. These cochleae were then submerged in 2% OsO_4 solution for 10 min and gently perfused through the openings to assure adequate fixation. Traces of OsO_4 were removed in two physiological saline rinses. The bony coverings of the cochleae were removed leaving the spiral ligaments intact. These cochleae were then submerged in ETHBr solution (10^{-3}M) for 10 min. Stained cochleae were dissected in 50% glycerol under a dissection microscope (Wild M-5). The basilar membrane and parts of the osseous spiral lamina were dissected from the cochleae and placed on glass slides. The tissue was immediately examined under ultraviolet (UV), phase contrast, and simultaneous UV-phase contrast microscopy using a Wild M 20 microscope.

Animals receiving acoustic stimulation were exposed to 4 kHz tones at 110 db SPL lasting up to 90 min. The stimulating signal was generated by an audiometer and was amplified by an Altec A340A amplifier. Signals were presented to the animals through an Electro Voice SP12 speaker in an Industrial Acoustics Company type 1202A sound treated room. Before stimulation test animals were anesthetized

with an injection of sodium pentobarbital (mg/kg i.p.) and taped to a stage in front of a speaker. The animals were prepared for histological study 24 hours after the termination of acoustic stimulation.

RESULTS

The fluorescent pattern produced by ETHBr (Fig. 1) clearly indicates the affinity of the stain for the nuclei of the cells of the organ of Corti. In Figure 1, the regular rows of fluorescing structures at the periphery (the right of Fig. 1) of the basilar membrane represent nuclei of outer hair cells (OHCs) and Deiters' cells. The inner sulcus area is seen to contain a number of nuclei of various shapes. The dark band in the center of the organ is associated with the bone of the osseous spiral lamina. The area of ETHBr fluorescence in the bottom center of Fig. 1 is the inner ear's membrane which was folded back during dissection. The organ of Corti during dissection shows a detail of the ETHBr fluorescence the same area as depicted in Fig. 1. The regularly spaced nuclei belonging to the outer row of nuclei at the periphery of the organ (top of Fig. 2) belong to Hensen's cells.

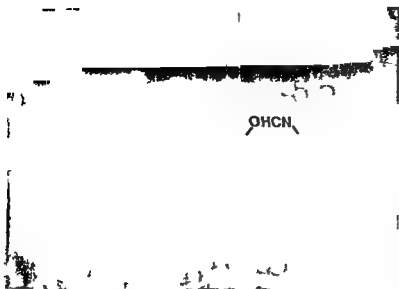


Fig 2 UV photomicrograph showing a detail of the ETHBr fluorescence in the same tissue as in Fig 1. OsO₄ fix on Magn fix on $\times 500$ OHCN outer hair cell nuclei HC Hensen's cell nuclei T tunnel IS inner sulcus

the Hensen's cells and other cells of the inner sulcus region as well as the spiral ligaments were removed during dissection. The area below the OHC nuclei, which is devoid of fluorescence is the tunnel (T). Some of the cells of the inner sulcus (IS) belong to inner ear cells (IHC) but it is difficult to differentiate these cells without the aid of phase contrast microscopy. An interesting band of ETHBr fluorescence extends inward (down

ward in Fig 2) from each of the first row OHC nuclei. This fluorescence might be illumination of the hair cell bodies by the fluorescing nuclei. In some preparations these fluorescent bands terminate in barely visible spots of fluorescence which correspond with the positions of cuticular plates of OHC.

Fig 3a is a phase contrast photomicrograph of the nuclear region of a row of OHC. The same cells photographed under UV light con

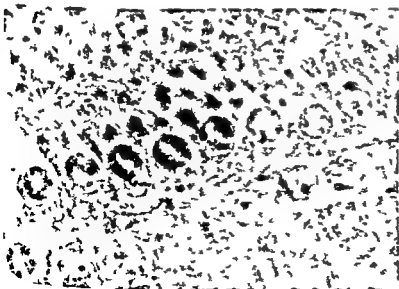


Fig 3a Phase-contrast photomicrograph of the nuclear region of a row of OHC. OsO₄ fix on Magn fix on $\times 1700$



Fig 3b UV photograph of the section in Fig 3a. ETH fluorescence. OsO₄ Magnification:



Fig 4a Phase-contrast photomicrograph of the reticular lamina. OsO₄ fixation. Magnification $\times 1200$. Arrows point to the cuticular plate of OHC referred to in Figures 4b and 4c.

ditions (Fig 3b) clearly show the ETH fluorescence in the nuclei. The fluorescence finding, which was a consistent finding in our preparations, suggests that ETH staining the interior structures of the nucleus. It is not clear whether these hyperfluorescent structures are masses of chromatin material or nucleoli. The row of nuclei which appear as blurred fluorescent images at the bottom of Fig 3b are Deiters' cell nuclei which are deeper in the tissue.

ETHBr fluorescence in cells exposed to acoustic stimulation

The fluorescence produced by ETHBr in tissue exposed to high intensity acoustic stimulation was carefully evaluated to determine if fluorescence was diminished. No observable difference in brightness of ETHBr fluorescence was found between cochlear tissue which had been exposed to acoustic stimulation and control tissue. Even in cells which were in an advanced state of degeneration, ETHBr fluorescence was unchanged.

A number of explanations for the lack of fluorescence loss are possible. First, improper stain concentration might have obscured a



Fig 4b Phase-contrast photomicrograph of the nuclear region of a damaged OHC in the same area as in Fig 4a. OsO₄ fixation. Magnification $\times 1200$

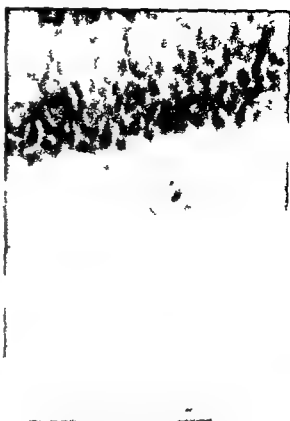


Fig 4c Simultaneous UV phase contrast photomicrograph of the nuclear region of the cell shown in Figure 4b. ETHBr fluorescence. OsO₄ fixation. Magnification $\times 1200$

use-induced changes in nucleic acids. Secondly the 24 hours between the end of acoustic stimulation and staining of cochlear tissue may have prevented observation of any reversible changes in nucleic acids lasting less than 4 hours and any long term changes which could not have appeared at the time of sacrifice. It is also possible that acoustically induced agitation of DNA and RNA helices may increase the number of available binding sites and in fact increase ETHBr fluorescence as suggested in a study by Nicolini et al (1974). Several studies have indicated that changes in nucleic acid concentrations do occur in the organ of Corti after acoustic stimulation (Nakamura 1967, Kluydens 1963, Vinnikov & Titova 1958, Ohhashi 1960, Kon 1964). It may be that two processes differing in sign

(i.e. an increase in ETHBr binding and a decrease in nucleic acid concentration) cancel each other and cause no loss in fluorescence.

Applications of ETHBr in cochlear research

The ETHBr fluorescence technique was found to be especially useful in detecting subtle damage in cell nuclei of tissue exposed to acoustic stimulation. ETHBr fluorescence was in many cases able to detect such damage even before the familiar gross alterations in cells and the reticular lamina characteristic of acoustic trauma appeared.

Because of its sensitivity the ETHBr technique appears to be an effective tool for damage assessment that has some advantages over the standard cytochromeoxidase technique, a common histological procedure which ac-



Fig 5a Phase-contrast photomicrograph of the cuticular lamina. O.O.S. Magnification $\times 1200$

counts for the status of hair cells by the appearance of the reticular lamina. The disadvantage in the use of cytochromeography can be seen in Fig 4. The OHC in this tissue would probably be classified as intact using the criteria of some cytochromeographic procedures. However, examination of the subcuticular region reveals a misshapen hair cell body even though the cuticular plate itself appears to be normal (Fig 4b). The same tissue

examined with the ETHBr technique (Fig 5a) shows a shrunken nucleus—a clue to possible pathology within this cell. A similar situation is depicted in Fig 5. The cuticular plate of this section of basilar membrane appears to be intact although some swelling and distortion is suggested in the third row of plates. A histological procedure which could account for the subcuticular and nuclear elements of these cells might well have

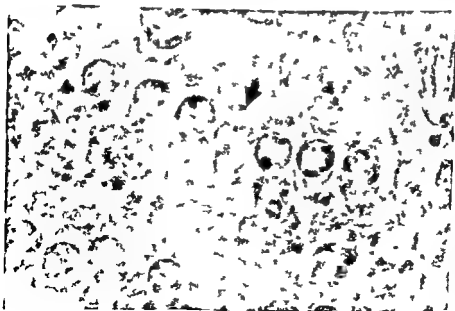


Fig 5b Phase-contrast photomicrograph of the clear region of OHC in the same area as shown in Fig 5a. The arrow indicates the shrunken nucleus. O.O.S. Magnification $\times 1200$



Fig 5c Simultaneous UV phase-contrast photomicrograph of the nuclear region of cells shown Fig 5a and 5b ETHBr fluorescence OsO₄ fixation Magnification $\times 1200$

oked the missing nucleus in an OHC which appears to have an intact cuticular plate. As seen in Fig 5a-c this damage is clearly evident with the aid of ETHBr fluorescence. If significant numbers of such abnormal hair cells are overlooked in an examination of the cuticular lamina the resulting high false negative error might seriously compromise the accuracy of the histology and any conclusions derived from it.

CONCLUSION

The present study employing UV and phase-contrast microscopy has determined that the fluorescing dots observed by Guth et al (1974) in cochlear tissue stained with TPHC-3 were cell nuclei. Photographic evidence presented in the present study suggests that ETHBr fluorescence was exclusively associated with cell nuclei in the organ of Corti and was remarkably similar to the pattern of fluorescence induced by TPHC-3.

Although acoustic stimulation did not produce a loss in ETHBr fluorescence similar to that in studies with other stains (Goldstein, 1973; Guth et al, 1974), fluorescent staining of nuclei in the organ of Corti proved to be a

valuable histological tool. Subtle forms of damage which were not readily visible in standard surface preparations of basilar membrane from sound treated animals were detected using the fluorescent staining technique.

SUMMARY

Guinea pigs were subjected to acoustic stimulation at 110 dB SPL for up to 90 minutes to determine if the fluorescence of ethidium bromide, a nucleic acid specific fluorescing stain, diminished in cochlear tissue. The loss of fluorescence previously reported by Goldstein (1973) and Guth et al (1974) in studies with other stains, was not observed. The use of ethidium bromide in surface preparations of basilar membrane was found to be especially useful in detecting subtle forms of noise-induced damage in the organ of Corti.

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ZUSAMMENFASSUNG

Meerschweinchen wurden akustischen Simulationen auf 110 dB SPL bis maximal 90 Minuten ausgesetzt, um zu bestimmen, ob die Fluoreszenz des Ethidium-Bromids eine nukleinsäure-spezifisch fluoreszierende Färbung im Schnechengewebe vermindert hat. Der Verlust der Fluoreszenz, früher bei Goldstein (1973) und Guth und Mitarbeitern (1974) berichtet, wurde während Untersuchungen mit anderen Färbungen nicht beobachtet. Die Benutzung von Ethidium Bromid an Oberflächenpräparaten der Basilarmembran wurde als besonders empfehlenswert für den Nachweis von geringen Änderungen in der gerauschinduzierten Beschädigungen der Schnecke erachtet.

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A STUDY OF THE VIBRATION OF THE BASILAR MEMBRANE IN HUMAN TEMPORAL BONE PREPARATIONS BY THE USE OF THE MOSSBAUER EFFECT

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Abstract Using the Mossbauer technique we have studied the vibration of the human basilar membrane and the malleus head in the sound frequency range 0.2-9.0 kHz at a sound pressure of 100 dB at the tympanic membrane. The displacement frequency response curves for the basilar membrane and the malleus head have similar shapes with a maximum at about 1 kHz. Below and above 1 kHz the curves have a slope of about 10 dB/octave and 0.0 dB/octave respectively. In addition the basilar membrane has a maximum displacement at a frequency dependent distance from the stapes. A simple hydrodynamic model for the cochlea is used to interpret the experimental data. A possible mechanism for the frequency resolution of sound by the ear and noise induced hearing loss is discussed.

The development of a satisfactory theory of hearing depends on measurements of the displacement characteristics of the basilar membrane in the cochlea. Such measurements were performed by von Békésy (1960) during the period 1924 to 1958.

Békésy put the stapes into sinusoidal vibrations with constant amplitude and measured by the use of a microscope under stroboscopic illumination, the displacement as a function of frequency at various points along the cochlear partition. Using human ears (cadavers) and the ears from many species of animals, a series of curves relating relative amplitude to frequency were obtained. As a function of frequency the amplitude for each point on the partition resembled a resonance curve. His experiments on human temporal bone preparations were restricted to the frequency region below 2 kHz and to very high sound pressure levels (~140 dB re 2×10^{-5} Pa). Johnstone &

Boyle (1967) applied the Mossbauer technique to measure the displacement amplitude of the stapes and the basilar membrane of living guinea pigs. Rhode (1971) used the same technique to measure the transfer ratio between the basilar membrane and the umbo of the malleus in squirrel monkeys. In both works the frequency response curves were found to have a positive slope (~10 dB/octave) on the low frequency side (below 1 kHz) and a rather steep negative slope (~-100 dB/octave) on the high frequency side (above 1 kHz). Kohlöffel (1972) introduced a new method to study the vibration of the basilar membrane. This method is based on the fact that when illuminated by a laser beam individual speckles on a surface become fuzzy when the surface vibrates with sufficient high frequency and amplitude. With this technique he measured the frequency response curves in living guinea pigs and found similar values for the slope as those of Johnstone & Boyle and Rhode. In dead specimens the values for the slope were found to decrease slightly with time after death.

The use of a capacity probe to measure the basilar membrane vibrations was first suggested by Békésy, but his probe was not small enough to give satisfactory spatial resolution. Wilson (1973) using a sub-miniature probe with a tip diameter of 0.2 mm, was able to measure vibrations down to 0.1 nm.

In the present work we have used the Mossbauer technique to obtain values for the ampli-

tude and phase for the head of the malleus and at various positions on the basilar membrane in human temporal bone preparations. An attempt is made to fit to the experimental data values calculated by using a hydrodynamical model for the cochlea. The implications of this model on the theory for hearing and on the loss of hearing shall be briefly discussed.

EXPERIMENTAL

The experimental arrangement consists of the following four parts: (i) an acoustical stimulus apparatus with which sound is applied to the external ear of a temporal bone, (ii) a Co-57 gamma (γ) source placed either on the basilar membrane or on the head of the malleus, (iii) an Fe-57 absorber (at rest) placed approximately 2 cm away from the source, and (iv) a γ detection system. Parts (ii), (iii) and (iv) comprise a Mössbauer spectrometer. The spectrometer, the acoustical stimulus apparatus and the preparation of the temporal bones are described in Sections A, B and C below.

The Mössbauer spectrometer

For the description of the Mössbauer effect on which this spectrometer is based we refer to a text book in nuclear physics. The working principle of the spectrometer is as follows. The source may emit and the absorber may absorb a 14 keV γ in a recoilless process. When the absorber is placed between the source and a detector the counting rate as a function of the relative velocity between source and absorber then comprises the Mössbauer spectrum. In our case both Co-57 and Fe-57 are embedded in a Rhodium metal foil in which case that spectrum will give one line of Lorentzian shape. The full width at half maximum, Γ , of that line was measured for our system to be $\Gamma = (0.360 \pm 0.005)$ mm/s.

The sources were cut from a 1 mm \times 1 mm \times 4 μ m Rhodium foil containing 30 mCi Co-57. In the measurements of the vibration of the basilar membrane and of the head of the mal-

leus, the sources had an area of about 40 μ m \times 50 μ m and of 200 μ m \times 200 μ m, respectively. The weights 0.2 μ g and 3 μ g of these sources are too small to significantly distort the movements of membrane and the malleus.

The absorber is a 4 μ m thick Rhodium foil with diameter 15 mm containing 5 atoms Fe-57. This is an optimum level of enrichment in Fe-57 giving a maximum dip in the Mössbauer spectrum without broadening and distortion of the line shape.

The detector is a scintillation counter (model MSP-1 from Elscint) with a 0.2 mm thick NaI(Tl) scintillator crystal shielded with a 0.2 mm thick Be window which permits passage of low energy gamma rays. The photomultiplier tube is an EMI type 9656. The counting efficiency for the 14.4 keV radiation is 100% and the resolution is approximately 40% when the tube is operated at a high voltage of 1 kV. The pulses from the detector are fed to an amplifier with a single-channel analyser which discriminates pulse heights resulting from the lines of the source pulse spectrum other than the 14.4 keV line.

II. Acoustical stimulus apparatus

To produce a sinusoidal stimulation a pure tone sine wave from an oscillator (Bruel Kjaer, B & K Beat Frequency Oscillator 1022) was used to drive an artificial ear (B & K, type 4215). The sound pressure was fed into the external auditory meatus through a plastic hose.

Through a circular hole made in the middle wall 2–3 mm from the ear drum, a microphone probe 4 mm in diam and 14 mm long was firmly cemented. Using this probe the sound pressure level was measured with a Brüel & Kjær inch condenser microphone with total probe length of 20 mm. With this configuration the output of the probe microphone at constant free field sound pressure level was constant within 0.5 dB for frequencies up to 1 kHz. Resonance occurred at 3.2 kHz. This produced a sensitivity enhancement of 25 dB at this frequency. To dampen this resonance peak

of steel wool was inserted into the 6 mm part of the probe nearest to the diaphragm of the condenser microphone. The condenser microphone had a linear behaviour up to 140 dB SPL.

Preparation of the temporal bones

In addition to the cementation of the microphone probe tube, described above, the preparation consisted of making access to the proper place on the basilar membrane. The malleus and part of the incus were exposed through an opening in the tegmen tympani. Through this opening it was easy to observe the movement of the head of the malleus under a microscope with stroboscopic illumination. It would have been preferable to use the displacement of the footplate of the stapes as a reference, but this structure is far less accessible for direct observation.

With a dental drill the bone covering the scala tympani was removed until a thin bony shell was left. A small hole ($\sim 0.5 \text{ mm}^2$) was usually made with a dissecting instrument. The hole was made at different places in the basal turn of the cochlea where the characteristic frequencies for middle and high frequencies are expected to lie. The fluid of the scala tympani was removed and the small source was placed on the basilar membrane with a glass needle. In most cases, when the scala tympani had been properly drained, the surface of the basilar membrane became quite sticky so that the source attached firmly to the membrane. If some fluid was left on the basilar membrane, the source would fasten to the surface membrane of the fluid instead, thereby causing the source to settle down improperly. When this happened, the source had to be removed and the process repeated. After the source had been properly placed, the scala was refilled with cerebrospinal fluid, which is similar to perilymph with respect to viscosity and surface tension. This refilling did not affect the position of the source to the basilar membrane.

A pipette with a capillary tube tip was used to refill the scala. The tip had to be guided

into the scala and the pipette pressed carefully in order to avoid the formation of air bubbles in the scala. It proved to be exceedingly difficult to remove such bubbles. The most successful method was to dip a small part of the bone into water and turn on an ultrasonic field for a short time. This treatment, in most cases, caused the bubbles escape to the surface.

The hole was then covered with a thin plastic sheet to keep the fluid level constant during the experiments. No hydraulic isolation was achieved with this plastic window.

To protect the temporal bone from drying and thereby preventing changes in the properties of sound conduction, the laboratory was kept at a relative humidity of 60 to 70%.

After the measurements of displacement and phase had been completed and the sources removed, the scala tympani was opened in order to measure more accurately the position of the source on the cochlear partition.

RESULTS

A Displacement amplitude

For a harmonic oscillation the displacement amplitude y_0 at a sound pressure p at the tympanic membrane is given by

$$y_0 = \frac{\Gamma}{2\pi f} \left(\frac{1}{(1-G)^2} - 1 \right)^{1/2} \quad (1)$$

Here, Γ is defined above, f is the frequency and

$$G = \frac{N(p) - N(0)}{N(\infty) - N(0)} \quad (2)$$

where $N(p)$, $N(0)$ and $N(\infty)$ respectively, are the counting rates measured at the pressure $p = 100 \text{ dB}$ (re $2 \times 10^{-5} \text{ Pa}$), with no stimulus and with the system heavily overstimulated ($p = 130 \text{ dB}$ at 1 kHz).

Values for y_0 are given in dB relative to 0.1 nm . To improve resolving power, the actual sound pressure p was sometimes more than 100 dB . The data were then extrapolated to $p = 100 \text{ dB}$, assuming the system to be linear.

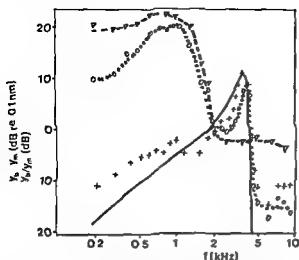


Fig 1 Frequency response of the displacement amplitude ∇ Malleus head (y_m) \circ basilar membrane (y_b) at $x=12$ mm from stapes $+$, ratio y_b/y_m . The solid curve represents calculated values for the ratio y_b/y_m , where y_s is the displacement amplitude of stapes

Experiments showed that in the actual pressure range the system was indeed linear and hence the normalization did not introduce significant errors

The displacement amplitudes y_m and y_b for the head of the malleus and the basilar membrane, respectively, were measured in the frequency range 0.2–9 kHz for 7 points on the basilar membrane between (9.9 ± 0.1) mm and (17.0 ± 0.1) mm from the stapes. For each source position, the response curves have similar shapes with a slight positive slope (about 10 dB/octave) below about 1 kHz and a steep negative slope (about 100 dB/octave) above that value. In addition to the broad peak at 1 kHz the curves for the membrane exhibit a resonance peak, the position of which depends on the value of x and is characteristic for the membrane itself.

A typical example is shown in Fig 1 with x at 12.0 mm. The curves through y_m and y_b are drawn by eye. In the same figure we have also plotted values for the transfer ratio y_b/y_m . The corresponding γ curve represents calculated values for the ratio y_b/y_s , where y_s is the displacement amplitude for the stapes. In the calculation we used a hydrodynamic model for the cochlea, as described in the last section.

Assuming γ_s to be proportional to y_s , we normalized the theoretical curve to the experimental values at the peak. We see that the calculated values reproduce quite well the position of the membrane resonance peak. Above that peak the experimental data approaches a constant value, whereas the theoretical curve approaches zero quite rapidly. This discrepancy is probably due to slow leakage or evaporation of the liquid in the middle ear. The mass between source and detector then decreases causing the transmission of 14 keV γ to increase with time. Since the reference rates $N(0)$ and $N(\infty)$ were measured at the start of the experiment the value of rate $N(p)$ and thus of the displacement amplitude y_b will increase with time. Independent experiments in which $N(0)$ was measured before and after the measurements of $N(p)$ indicated the presence of such a systematic error. This error is only serious at the highest frequencies and varies from sample to sample. In the example shown in Fig 1 the error as represented by the tail of the membrane response curve is only about 0.02 nm. Sometimes the tail was as high as 0.1 nm.

The measured values for the displacement amplitudes varied from one preparation to another.

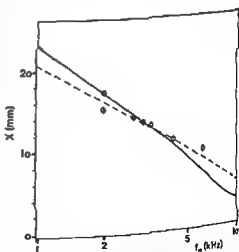


Fig 2 Relation between the frequency f_m and the position x on the basilar membrane of maximum displacement amplitude. — Calculated values using our modified Greenwood's formula (eq 3)

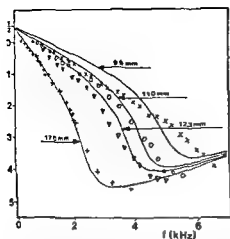


Fig. 3. Phase frequency response at 4 different positions x along the basilar membrane. The points are experimental values for the phase difference between the basilar membrane and the head of the malleus. The curves represent calculated values for the phase difference between the membrane and the stapes. Values for the position x are given in the figure.

A variation in sound transmission, measured as volume amplitudes at the round window, is also found by Elperm & Andersen (1965) and Gundersen (1971). Gundersen (1971) found in 32 preparations that the patient's age at death was without significance for the sound transmission; the lapse of time between death and recordings being insignificant within wide limits. Most people have experienced acute otitis in the middle ear at least once. Small mechanical loss on account of adhesion after such infections may be the cause of the observed variations of the displacement amplitudes.

We were not able to investigate the possible effect of the size of the opening of the scala tympani. To do this properly, measurements at various sizes ought to have been performed on the same specimen, which was not possible due to the time factor.

The calibration of the probe microphone is another experimental problem. The calibration procedure was carried out in a sound field at 100 dB SPL. In the sound measurements on the external meatus the probe tip was placed approximately 3 mm from the drum. This distance represents ap-

proximately 10% of the pressure wavelength at the highest frequencies or approximately an error of 0.5 dB in the pressure at 5 kHz and 2 dB at 10 kHz. No correction for these errors was applied.

In addition to the opening, the source itself might represent a disturbance to the movement of the basilar membrane. The mass of the source is approximately $0.1 \mu\text{g}$ which is much less than that of the element of the cochlear structure upon which the source rests. If the source is in good contact with the surface of the membrane, that is, if the stiffness of the coupling between membrane and source is high, the source will follow the membrane movement at high frequencies. The only evidence that this coupling was strong is that the source was always found to be in the same place after the measurements had been completed.

Due to these errors (discussed above) we believe that the experimental values for the transfer ratio can only locate the characteristic frequency, f_m , at which the resonance curve has its maximum. In Fig. 2 we have plotted on a semilog scale the position x of the source as a function of f_m . The solid curve represents calculated values using the hydrodynamic model and the broken line represents the following semiempirical formula according to Greenwood (1961):

$$x = 35 - 16.7 \log_{10} (0.006046 f_m + 1) \quad (3)$$

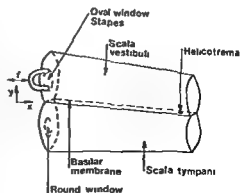


Fig. 4. The uncoiled simplified model of the cochlea used in the calculations.

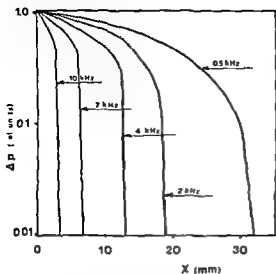


Fig 5 Calculated values for the pressure difference Δp across the basilar membrane as a function of the distance x from stapes at various frequencies

B Phase response

Phase response measurements were carried out in the following way. Each time the signal from the condenser microphone passed through zero (on positive going) a trigger signal at the multiscaler to channel one. Clock pulses with calibrated repetition time advanced the channel address within the period of the sound stimulus, giving a histogram with transmitted counting rate as a function of phase. In Fig 3 are shown experimental results for the phase difference ϕ between the basilar membrane and the head of the malleus for four different positions along the membrane. As seen from Fig 3 all the phase data start at $\phi = +\pi/2$. This is based on the assumptions that at low frequencies (i) the displacement of the membrane must lead that of the stapes by $\pi/2$ (Rhode 1971) and (ii) the head of the hammer and the stapes are in phase. The curve drawn in the figure is theoretical and will be discussed in the next Section.

The uncertainties in the experimental values of the phase of the basilar membrane relative to the phase of the middle ear structures are smaller than those of the displacement, hence the phase measurements are more reliable.

DISCUSSION

A Independent resonator hydrodynamic model

The experimental data were found to be produced quite well by calculated values in a model for the cochlea as suggested by Zwislocki (1953). Typical examples of the fit have already been given in Figs 1, 2 and 3. We will in the following only give a qualitative description of this model. For a more complete account, we refer to Zwislocki (1953) and Stein (1976).

The model is based on the following assumptions:

- 1) The coiling of the cochlea is unimportant.
- 2) The fluid in the cochlea is ideal and incompressible.
- 3) The walls of the cochlea are rigid and introduce resistance to the flow.
- 4) At a position x on the membrane the area of a cross section of the scala tympani is 1/10 to that of the scala vestibuli and decreases slowly with increasing x .
- 5) The membrane is a passive element and is regarded as consisting of a set of independent resonators with no mechanical coupling and having displacements which are small relative to the cross dimension of the scalas.

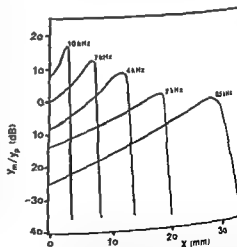


Fig 6 Calculated values for the ratio y_m/y_p between displacement of the basilar membrane and of the stapes as function of the distance x from stapes at various frequencies

A schematic drawing of the cochlea is given in Fig. 4. Using this model we have calculated the pressure drop Δp across the membrane and the ratio y_m/h_x between the displacement amplitude of the membrane and the stapes as a function of x at selected frequencies. The results are shown in Figs. 5 and 6. We see from

Fig. 5 that at constant frequency Δp decreases with increasing x . Furthermore, as the frequency decreases, the length of the membrane which is exposed to a pressure difference increases. The curves for the displacement ratio given in Fig. 6 have shapes typical of resonance curves.

A qualitative description of the oscillations in the cochlea is as follows. The vibrations of the footplate of the stapes at the oval window are transmitted in the liquid in the scala vestibuli and the scala tympani to the round window. At extremely low frequencies the whole mass of the liquid in the scalas participates in the movement resulting in a pressure drop along the whole length of the membrane. As the frequency increases the mass of the participating liquid and hence the length of the membrane which oscillates decreases. In addition to the mass, the resistance of the liquid together with the elastic properties of the membrane and the structure of the inner ear influences the position of the displacement maximum and oscillation characteristic of the membrane.

The results of the calculations as given in Figs. 5 and 6 shall now be discussed in relation to the audio frequency resolving power and hearing loss.

Frequency resolving power

The tectorial membrane and the basilar membrane are anchored at their inner edges at spatially separate points, thus producing a shearing force on the hairs when the basilar membrane vibrates. The firing level of a nerve is probably set by a lower level for the displacement amplitude. It is interesting to note

that according to our results at an input sound pressure level of 100 dB the maximum displacement is of the order of 100 nm. Assuming a linear relationship between displacement and sound pressure the displacement at the threshold of hearing is of the order of 10^{-3} nm which is far less than the thickness of a cell membrane (~ 10 nm).

Of course such a straight extrapolation is not necessarily correct. However, this indicates that the firing level amplitude is so small that the basic mechanism in hearing is molecular in nature. The combined response of the middle and inner ear as measured for points of the basilar membrane between 9.9 mm and 17.0 mm shows that the displacement amplitude at frequencies around 1 kHz exceeds the displacement amplitude at the frequency corresponding to the maximum ratio between basilar membrane and head of the malleus displacement. The displacement ratio increases slowly with x to a maximum value, above which it falls rapidly to zero. For a sound containing several well separated frequencies the composite displacement ratio curve will contain as many peaks and steep portions as there are frequencies. The positions of the steep portions are much better defined than those of the peaks. Hence it is more likely that it is the spatial derivative of the displacement amplitude and not the amplitude itself that determines the firing level of the nerve.

C. Hearing loss

The results given in Figs. 5 and 6 might explain why noise induced hearing losses appear first in the higher frequency region almost regardless of the frequency components of the noise. We shall illustrate this with an example. Suppose the noise has a frequency of 0.5 kHz. According to Fig. 6 and the discussion above, the ability to resolve such a frequency depends on the nerves on the membrane in the region around $x \approx 30$ mm. As seen in Fig. 5 the largest pressure differences (and thus displacement) are, however, at the lowest values of x .

Hence, damage to cochlea is most likely to occur in the region of the basilar membrane where the higher frequencies are resolved.

ZUSAMMENFASSUNG

Mittels der Mößbauer-Technik haben wir die Bewegungen in der humanen Basilar membrane und in dem Caput Mallei studiert. Lautdruck ist von 100 dB im Frequenzgebiet 0,2 kHz bis 9 kHz angewandt. Die Kurven der Bewegungsamplituden in der Basilar membrane und dem Caput Mallei haben ein gleiches Aussehen mit Maximum etwa 1 kHz. Unterhalb und oberhalb 1 kHz fallen die Kurven mit respektiven 10 dB pr. oktav und 100 dB pr. oktav. Außerdem hat der Ausschlag der Basilar membrane eine maximale Bewegungsamplitude abhängig von der Frequenz und dem Abstand des Stapes. Ein einfaches hydrodynamisches Modell für Cochlea ist angewandt, um die experimentellen Data auszulegen. Eine mögliche Erklärung, warum wir Lärmschaden in besonderem auf speziellen Frequenzen bekommen, ist diskutiert.

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DIAGNOSIS AND SURGERY OF ACOUSTIC TUMOURS

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Abstract In the diagnosis of acoustic neuromata the stapedial reflex test, the forward vs reverse Bekésy test, loudness balance test, and the adaptation test have all been found superior among the special tests used in pre-tumour diagnosis. Conventional X-ray studies of the internal acoustic meati are always performed, followed by tomograms and in suspected cases by meatocisternography. In the present series translabrynthine total removal was performed in 21 cases, a planned decompression in 5, while in 2 the removal was subtotal. Only permanent facial paralyses arose, one corrected by zosteric plasty and the other by facial hypoglossal anastomosis. Otolologists are urged to regard every unilateral perceptible hearing impairment as being caused by a tumour until proven otherwise.

Otolologists have in recent years urgently felt the need for early recognition of acoustic neuromata. Earlier, this diagnosis was not considered seriously until associated symptoms were present, e.g. reduction of corneal sensitivity or atactic symptoms, in addition to impaired hearing and vertigo. The change of attitude is due to the work of William House and his associates (1964, 1968) who persisted in perfecting the technique of translabrynthine tumour removal until the results convinced NT surgeons all over the world that for small and medium sized tumours this method is unquestionably superior to the neurosurgical approach.

MATERIAL AND METHODS

This report is based on a material comprising 42 cases of acoustic neuroma and 36 cases of Meniere's disease to allow comparison of audiological data. In addition 29 cases are in-

cluded in which a tumour was suspected but positive contrast meatocisternography showed normal findings. The ages of the patients ranged from 21 to 76 years (mean 47 years) in the acoustic neuroma group, from 23 to 63 years (mean 40 years) in the Meniere group, and from 11 to 63 years (mean 43 years) in the group of sensorineural loss with retrocochlear characteristics. For preoperative audiologic evaluation, hearing tests for pure tone and speech audiometry, stapedius muscle reflex threshold measurements, Fowler's loudness balance and interrupted vs continuous tone Bekesy tracings as well as forward-reverse continuous tone tracings were carried out. Possible pathological threshold was studied using the 3 min fixed frequency continuous tone stimulation.

In the vestibular evaluation, tests for spontaneous and lateral gaze spontaneous nystagmus were performed and the caloric responses were recorded using water stimulation at both 44° and 30°C.

In roentgen examination projection through the orbit, the Stenvers projection and skull base views were obtained. Whenever indicated these were supplemented by tomographic examination of the internal meati, by positive contrast meatocisternography using 1 ml of contrast medium (Myodil) and 50 mg of cortisone, suspended in 1 ml spinal fluid. In certain cases, finally, pneumoencephalography was carried out. Surgical exploration of the internal acoustic meatus was performed in 28 cases of acoustic neuroma and in all 36 patients with Meniere's disease.

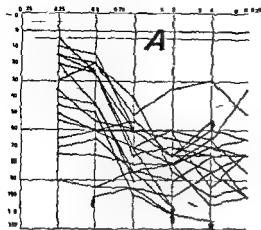


Fig 1 Individual air conduction curves in 23 neuroma cases (A) in 36 ears with Meniere's disease (B) and in

29 patients with sensorineural hearing loss without clear characteristics but normal meatocisternography

RESULTS

Preoperative evaluation

Shape of audiogram Fig 1 shows the air-conduction audiograms of 23 cases of verified acoustic neuroma (9 were preoperatively deaf). The air-conduction curves for the group of 36 patients with Meniere's disease are presented in Fig 2 and those of 29 patients with audiological impressions of retrocochlear lesion but with normal filling of the meatus in meatocisternography in Fig 3. The general pattern in the tumour cases was downward sloping air-conduction curve, while the Meniere patients had flatter curves and the third group a mixture of both.

The results of the various audiological tests included in our test battery are summarized in Table II. Totally deaf ears when testing by air conduction appeared in all groups but the number of ears also deaf for speech was significantly higher in the neuroma group than in the two other groups. Negative loudness recruitment appeared only seldom in Meniere's disease (27%) but significantly more often in the neuroma group. In this series adaptation exceeding 30 dB at 500 Hz did not occur in Meniere cases, whereas in tumour cases it was observed in 26% and in the group of sensorineural deafness in 25%. Adaptation at 2000

Hz brought the figure to 20% even in Meniere group.

Stapedius reflex threshold test was significantly more often negative (70%) in the neuroma cases than in Meniere's disease (17%) and the cases grouped sensorineural (25%). Conventional Bekesy testing did not show significant differences between the groups, whereas the forward vs reverse technique gave significantly more positive data in neuroma cases (63%) than in Meniere's disease (4%).

The probability of arriving at the correct diagnosis by the various tests employed in the test battery was next evaluated. The validity of each test was measured on the basis of accuracy with which the test result had predicted neuroma in the cases where the suspicion was verified. The outcome is given in Table III. The tests are arranged in order of decreasing validity and in this series the stapedius reflex test together with the forward reverse Bekesy technique, emerged as the ones that most often accurately predicted neuroma diagnosis.

Positive contrast meatocisternography Table IV gives the results of the contrast study divided into groups on the basis of the evaluation of the internal meatus on tomography. Among 24 cases with normal roentgen



Fig 2 Normal meatocisternography X rays of left ear directed through the orbit (left) and axially (right). Arrows point to contrast medium in meatus

Of 3 tumours were found on 17 examinations by positive contrast meatocisternography on the one hand while on the other hand out of 16 contrast studies with pathological plain roentgenographs 15 were positive for tumour

Fig 2 shows our normal roentgenographs after filling the meatus the X ray having been directed through the orbit through the maxillary antrum and axially. Examples of various abnormal results are seen in Figures 3 and 4.

Pneumoencephalography was performed on



Fig 3 Absent filling but contrast medium droplets (arrow) near the cochlear wall (left). Right: a tumour extends 1 cm into the angle. Arrow points to the medial front of contrast medium around the tumour

Table I *Audiologic findings in three different disease groups*

Audiologic findings	Neuroma (N=32)		Meniere's disease (N=36)		Sensorineural X ray normal (N=29)	
	N	%	N	%	N	%
Flat audiogram	11	34	32	89	21	72
Deaf ear, air conduction	10	31	8	22	2	7
Deaf ear, speech	17	53	10	29	8	28
Discrimination <60%	23	72	21	62	11	38
No recruitment (Fowler)	13	65	7	27	11	38
Pathological adaptation						
500 Hz	5	26	11	30	7	25
2 000 Hz	14	63	5	20	12	43
Stapedius reflex threshold >120 dB	14	70	6	26	6	25
Békésy Type III or IV positive	3	15	6	23	7	26
Forward vs reverse tracing positive	12	63	1	4	9	31

3 patients as the first procedure and as the findings were positive in all, meatocisternography was not undertaken.

Complications of meatocisternography were seen in 3 of the 68 cases and included a high fever for 4 to 5 days, a stiff neck but no indications of a bacterial contamination. All reactions subsided without sequelae and were, in our opinion, caused by some oversensitivity to the contrast medium on the part of the patient. Symptoms of the cauda equina area irritation did not appear.

Cerebrospinal fluid (CSF) protein

Table V gives the results of total determination in the three separate groups. Only the values exceeding 100 mg/% seem to be concentrated in the tumour group but the majority of neuroma cases show quite low protein values.

Treatment of neuroma cases

The established method of treatment is tumour removal whenever possible. Our arrangement is to remove all small and medium size tumours in the ENT Department. If in doubt we obtain a pneumoencephalogram and if a tumour is found to leave a positive impression on the pons, the case is remitted to the neurosurgeon. If, on the other hand because of the patient's poor general condition or age, a neurosurgeon does not consider the neurological approach feasible, we perform a thorough decompression in the ENT Department without attempting total removal.

The surgical technique includes the translabrynthine approach with total mastoidectomy, desceletonization of the internal meatus and removal of bone from the sigmoid sinus, the cerebellopontine cistern. Tumour is then removed from the meatus and the

Table II *Results of diagnostic tests*

Test results	Neuroma cases (N=32)		Meniere cases (N=36)		Normal X ray (N=29)	
	N	%	N	%	N	%
Deaf ears	10	31	8	22	2	7
All tests						
retrocochlear lesion	3	15	0	0	1	3
cochlear lesion	2	10	3	11	7	24



Fig. 4 Filling defect produced by a 2 cm tumour (left) and by a 3 cm tumour (right). Arrows point to the medial extent of contrast medium around the tumour.

have identified in its peripheral end. Intracapsular removal is continued towards the pons and by working from different directions, the tumour capsule is gradually removed together with the tumour. The connecting veins to the capsule are coagulated as they appear, and thus bleeding vessels are avoided in advance. The cerebellum and the pons are protected by cottonoids which are inserted around the tumour as soon as the intracapsular removal has been adequate to allow shifting the tumour

margins to the centre of the operating field. In cases of large tumours, great care has to be exercised in separating the tumour from the IX–XI cranial nerve group, from the V nerve, from the anterior inferior cerebellar artery and from the pons. Once the central part of the facial nerve has also been identified, the rest of the tumour can gradually be separated from the whole nerve. With increasing experience even unexpectedly large tumours can safely be removed via the translabrynthine route.

Table III Neuroma diagnosis. Accuracy of audiological tests

Test	Per cent accurate
Stapedius reflex >120 dB	71
Forward vs reverse tracing positive	71
Pathological adaptation >30 dB at 2 000 Hz	66
at 500 Hz	54
Loudness balance test	63
Speech discrimination >60%	60
Békésy type III or IV positive	54

Table IV Evaluation of meatal tomography in cases suspected to have acoustic neuroma related to results of cisternography

Evaluation of meatal tomography		Cisternography	
		Negative	Positive verified by surgery
Normal	24	14	3
Questionable	22	18	2
Suspicion	15	12	3
Pathological	17	1	15
Total	78	45	23

Table V CSF protein in three different disease groups

CSF protein (mg/%)	Meniere's disease	Sensorineural hearing loss	Acoustic neuroma	
<50	8	23	0	
50-100	11	16	4	
>100		2	8	
Total number of patients studied	10	41	21	72

We remove the tumour tissue under slight hypotension and allow the blood pressure to return to normal when removal is completed. All possible bleeders are cauterized by applying bipolar cautery, or weak current unipolar cautery. The operative mastoid cavity is filled with fat taken from the abdominal area. If the tumour has been small and the cerebellar dura kept nearly intact, no fixing of the fat is required. If the dura has been split up to the sigmoid sinus, the innermost large piece of fat is fixed with catgut suture to the dural margins.

Table VI shows the results during the years 1974 to 1976. Total removal was successful in 21 cases. A permanent facial paralysis occurred in one case, later treated with masseter-masseter transposition. In 11 cases there was a temporary facial paralysis lasting from 2 weeks to 6 months and in 19 patients the facial nerve function remained intact the whole time. In 2 cases the tumour could not be safely removed by the translabyrinthine approach and a second stage was done in the Neurosurgical Department. One of them lost the facial nerve function completely but regained it in a very satisfactory manner after our hypoglossal-facial anastomosis 3 months after tumour removal.

Decompression was performed in 5 cases with total removal of the tumour from the internal acoustic meatus, and a large part of the intracapsular tumour. In 4 patients a dramatic

improvement occurred and they have been quite capable of taking care of themselves. In the fifth, a man of 76 years, surgery was attempted without complications and the patient awakened normally but developed an intracerebral hemorrhage 4 hours later and died within a week without regaining consciousness.

All 21 patients with total removal in the ENT Department regained normal working capacity within 2 to 3 months.

During the period 1973 to 1975 surgery was performed on 44 patients in the Neurosurgical Department. The majority of these were large tumours causing a number of symptoms in associated structures. During the immediate post-surgical period the mortality was 3 at home 2 (11%). The anatomic continuity of the facial nerve could be preserved in 18 but 8 (18%) regained the function. Fifteen patients could return to work after surgery (Toussaint 1977).

COMMENT

Unfortunately, audiological diagnosis of acoustic neuroma remains difficult and at present there are no tests not even a test battery that can give a definite confirmation (minimum 90%). Thus, in addition to the rarity of these tumours, causes a delay in diagnosis, which may be very unfortunate from the operative point of view, the tumour grows into dimensions threatening the vital centre removal.

We have made it a rule to start examining

Table VI Results of translabyrinthine surgery

Operated cases	28
planned decompression	5
subtotal removal	2
total removal	21
Facial paralysis	
permanent	1
temporary	8
intact function	19

diagnosis or exclusion of the tumour, if there is a slight unilateral perceptive impairment of hearing often combined with tinnitus and a somewhat lowered excitability in vestibular testing. At this stage we have the inner acoustic meatus X-rayed and apply part of the test battery referred to above. On the basis of the data obtained the pure tone and speech tests are supplemented first with the stapedius reflex threshold test and then with forward reverse Bekesy test (Palva & Jauhainen 1976). If these show recruitment and overlapping curves and the meatal X rays are normal, provisional diagnosis of inner ear deafness is made and possible treatment initiated. When there is some X ray suspicion of meatal enlargement or loss of bone density, binaural loudness balance and 3 min threshold adaptation tests are performed. The routine Bekesy interrupted continuous tone tracing has in our experience given less information and has later been omitted from the test battery as have the SISI tests. Stapedius reflex decay tests (Anderson et al., 1970) have been conducted in some cases where reflex threshold could be recorded. In all three groups both normal and pathological decay could be observed. Unfortunately the threshold for stapedius reflex was higher than 120 dB and no reflex decay could be measured in many cases of acoustic neuroma. On account of great work load in the audiological department some test selection has become more and more pressing.

Every case with unilateral perception deafness is followed once or twice a year and if the hearing loss or vestibular excitability shows further change, studies aimed at tumour exclusion are intensified. The same applies to cases with continuously deteriorating bilateral perceptive impairments.

As soon as it seems warranted meato-cisternography with positive contrast medium is undertaken. In our series of 68 examinations the side effects have been few and none serious, and no signs of radiculitis in the cauda area have appeared. In this respect our results are similar to those reported by House and the

risk of complications caused by the contrast medium seems to be minimal with the present preparations.

Interpretation of the positive contrast meato-cisternography always is easy if the tumour has already filled the inner meatus, or extended into the cerebellopontine cistern. Difficulties have been encountered in the evaluation of partially filled meatus where part of the canal remains empty of the bulk of the contrast medium. However, if small droplets are seen at the bottom of the meatus at the entrance of the nerves to the cochlea, we have interpreted the meatus to be free of tumour and in 4 such cases where vestibular and cochlear nerve neurectomies were performed there was no tumour but an increased amount of arachnoid mesh. Nevertheless, if no surgery is indicated, careful following of the patient should continue and if objective signs increase, a new cisternography can be undertaken later.

Surgery of these tumours is always to be regarded as a major intervention and where and by whom it should be made depends upon the local situation. In small countries like Finland with a population of 5 million, about 30 to 40 cases should be diagnosed each year. This small number necessitates regional concentration as otherwise no one will have sufficient experience in this surgery. In Finland 90% of the cases are operated on in Helsinki, large tumours by agreement in the Department of Neurosurgery and the small and medium sized tumours in the ENT Department. Close collaboration is essential in the selection and evaluation of the patients and should be done in the best interest of the patient.

In ENT surgery the translabyrinthine approach affords excellent visibility for small tumour removal but careful anatomic dissection with application of bipolar cautery makes possible total removal even of large tumours. However, in our experience the large opening to the field practised by the neurosurgeon adds to the safety of the procedure. The surgical technique as such as well as the possible com-

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Unfortunately, audiological diagnosis of acoustic neuroma remains difficult and at present there are no tests, not even a test battery that can give a definite confirmation (minimum 90 %). This, in addition to the rarity of these tumours, causes a delay in diagnosis, which may be very unfortunate from an operative point of view. The tumour grows into dimensions threatening the vital centres.

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temporary	8
intact function	19

PULSE VOLUME RECORDINGS IN OUTER EAR CANAL IN PULSE SYNCHRONOUS TINNITUS

A Comparison between Ears with Glomus Tumour, Serous Otitis Media and Normal Ears

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Abstract With the aid of a volume flowmeter it is possible to record pulse synchronous volumetric changes in the outer ear canal. In 7 ears with glomus tumour in the tympanic cavity and in 5 with serous otitis media such changes were larger than in 125 persons with a normal middle ear. By changing the ambient pressure in a pressure chamber and instructing the patients not to swallow the drum can be pushed inward or outward. In all the cases of glomus tumour studied the pulse volumetric change was considerably affected when the drum was pushed inward or outward. In the normal patients the change was only small and in the 5 cases of serous otitis media there was no change at all. This means that the pulse volume changes in normals are generated mainly by the vessels in the outer ear canal.

This paper reports on the first of a series of investigations into tinnitus of middle ear origin. Pulse-synchronous tinnitus is mostly caused by conditions outside the middle ear cavity, i.e. arterial hypertension or vascular anomalies but can also emanate from a glomus tumour in the tympanic cavity. Visible tumour behind an intact ear drum, conductive hearing loss and pulse synchronous tinnitus are the three most usual manifestations of this disease (Spector et al., 1975). A sign easy to demonstrate and sometimes diagnostically useful is that described by Brown in 1953, i.e. visible diminishing and disappearance of the pulsations and paling of the area affected by the tumour on inspection through an airtight microscope under increasing pressure in the outer ear canal.

Ingelstedt et al. (1967) studied middle ear mechanics with a flowmeter connected airtight to the outer ear canal and open to the ambient

pressure. Movement of the tympanic membrane creates a flow of air which is recorded in the flowmeter. The flow is automatically time integrated, whereby the volume displacement of the tympanic membrane can be recorded directly. The equipment is very sensitive and with high amplification pulse-synchronous volume changes are always recorded—even in normal ears. The question arose whether these pulse volume changes are caused by variation of the caliber of the vessels in the skin lining the ear canal, in the tympanic membrane or in the middle ear mucosa and transmitted via an intact ear drum, thus making them measurable. We therefore selected ears with a histologically verified glomus tumour and compared the pulse volume changes in the outer ear canal with normal ears. Ears with serous otitis media were also studied because fluid is incompressible and thereby directly transmits the pulsations from the middle ear mucosa to the tympanic membrane.

METHOD

Fig. 1 shows a block diagram of the recording equipment. It consists of a flowmeter connected airtight to the external ear canal and open to the atmospheric pressure which was kept constant. The flow generated by pulsations of the vessels (V_{pulse}) is recorded in the flowmeter. After amplification and integration of the flow signal it is possible to record the variation of the pulse synchronous volume from the ear canal and the middle ear (ΔV_{pulse}) directly by an ink jet recorder.

The pressure chamber equipment is used to record the pulse volume changes at different positions of the drum (Fig. 2). With this equipment the ambient pressure around

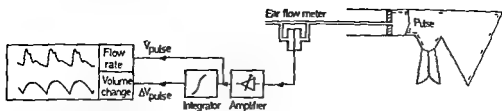


Fig 1 Equipment for recording flow rate (V_{pulse}) and volume changes (ΔV_{pulse}) in the external ear canal. For details see text

the person can be varied (by up to ± 15 cm H_2O) with a consequently varying degree of convexity or concavity of the tympanic membrane when the eustachian tube is kept closed. The pressure of the chamber (P_{ch}) is recorded by a differential pressure transducer.

With the flowmeter it is possible to record the volumetric displacement of the tympanic membrane (ΔV_{tm}) when it is exposed to different pressure gradients (Elner et al., 1971a).

The accuracy with which the variation of the volume is recorded is checked by means of an identical flowmeter connected to an adjustable reference volume. In the situation of volume balance between the two flow systems, the flows caused by the ambient pressure variations, V_{ec} and V_{ref} , are identical (Fig. 2). After subtraction in the differ-

tial amplifier the isolated recording of the flow is now only by volumetric changes in the ear canal and those caused by volume displacement of the tympanic membrane ΔV_{tm} . The sensitivity of the flow amplifier is adjusted in such a way that an integrated air flow volume of $0.01 \mu\text{l}$ through the flow meter causes a deflection of 1 mm by the inkjet recorder. The gas passing through the resistor is calibrated before and after each experiment with the aid of an airtight syringe containing $1 \mu\text{l}$. Reading accuracy $1 \mu\text{l} \pm 5\%$.

Linear response of the flowmeter system 0–500 $\mu\text{l/s}$. Dynamic sinusoidal response flat over 0–11 cps. Transient response 95% 20 msec. (For detailed data see Elner et al., 1971a).

An xy recorder was used for recording the volume

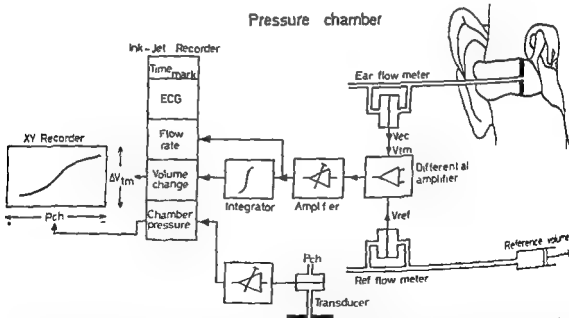


Fig 2 Equipment for recording volumetric changes in the ear canal (ΔV_{pulse}) and volume displacement of the tympanic membrane (ΔV_{tm}) in the pressure chamber. V_{tm} The air flow velocity through the ear flowmeter caused by the movement of the tympanic membrane. V_{ec} The air flow velocity through the ear flowmeter

caused by compression or expansion of the gas in the ear canal and in the flowmeter system by changing the ambient pressure.

V_{ref} The velocity of the flow through the reference flowmeter caused by compression or expansion of gas in the reference system by changing the ambient pressure.

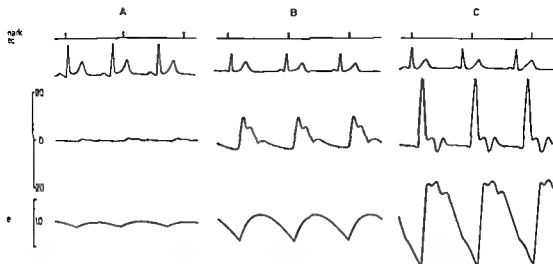


Fig. 3 Typical recordings in a normal case (A) in serous otitis media (B) and in glomus tumour (C). Pulse flow

(V_{pulse}) pulse volume change (ΔV_{pulse}) in the ear canal and ECG are recorded simultaneously

on of the tympanic membrane with the eustachian closed as a function of the change in the pressure number (P_{en}) i.e. the volume pressure relationship of the tympanic membrane system (see Elner et al. 1971b).

CLINICAL MATERIAL

The material consisted of 6 patients (7 ears) with a histologically verified glomus tumour and 5 patients (5 ears) with serous otitis media.

The cuff was placed in position in the bony part of the outer ear canal and checked for leakage. The catheter was hermetically connected to the measuring device on which the pulse flow (V_{pulse}) pulse volume change (ΔV_{pulse}) were recorded with the drum in controlled neutral position and on some patients an ECG was also recorded simultaneously. Nine of the 125 normal ears, 2 of the 7 ears with glomus tumour and all the cases of serous otitis media were also pulse tested with the drum in different positions. These positions were produced in the pressure chamber with the eustachian tube closed. The test procedures were always performed with the patient sitting.

RESULTS

Fig. 3 shows typical recordings in a normal control, in serous otitis media and in glomus tumour. The flow (V_{pulse}) recording resembles that of an artery pressure pulse curve and is related to the ECG recording. The pulse volume change in the ear canal (ΔV_{pulse}) in the normal is 0.2 μl , in serous otitis media 0.54 μl and in the glomus case, 1.5 μl .

Fig. 4 shows the pulse volume changes recorded in the ear canal in normals (125) and in patients with glomus tumour (7 ears) with the drum in controlled neutral position. The difference was statistically significant ($p < 0.001$). The 5 patients with serous otitis media showed pulse volume changes between 0.46 and 0.64 μl . In patients with glomus tumours and serous

shown in Table I

PERFORMANCE OF TEST

After microscopic inspection of the test ear a polyethylene catheter (PE 190) with an individually chosen foam rubber

Table 1 Age distribution of the material

Age	No. of ears		
	Normal	Glomus tumour	Serous otitis media
70	5		1
60-70	88		
50	20	1	2
40-50	11	2	1
30-40	4	4	1
20-30	125	7	5

Table II Symptoms and findings in the cases of glomus tumour of the middle ear

Case	Symptoms and findings	Hearing level dB bone + air*	Preop ΔV_{pulse} (μl)	X ray T Tomography A Arteriography J Jugulography	Operation
I Woman age 53 Left	Pulse synchronous tinnitus 1964 Susp tumour in post inf quadrant	13/37	1.3 Neutral	T and J Neg	1967 expl tympanotomy. Tumour a lower part of tympanum fixed here to drum
II Woman age 56 Left	Pulse synchronous tinnitus 1962 Susp tumour in ant and inf quadrant not in contact with ear drum	10/13	0.56 Neutral	A and J Neg	1969 expl tympanotomy. Small tumour just behind and part of eustachian tube
III Man age 45 Left	Pulse synchronous tinnitus 1968 Susp tumour in post inf quadrant	10/27	0.4 Neutral 0.42 Inward 0.22 Outward	A Conglom of vessels in hypotymp and on promontorium	1974 tumour mass of tympanum. Ext of bony canal and extirp of tumour placement of the wall + myringoplasty
IV Man age 47 Left	Pulse synchronous tinnitus 1964 1968 blue tumour except in ant sup quadrant	10/60	3.5 Neutral	J Susp glomus tumour A Tumour with vessels from a pharyngeal asc	1968 extirp of large tumour + radical mastoidectomy
V Woman age 55 Bilat	Pulse synchronous tinnitus bilat 1968 Both ear drums reddened	Right 38/38 Left 23/26	Right 2.4 Neutral Left 1.2 neutral	J Neg bilat A Susp tumour bilat	1969 expl tympanotomy bilat Right 2 tumours Left 1 tumour
VI Man age 32 Left	Pulse synchronous tinnitus + dysphagia 1972 Red tumour in the ant + inf quadrant Paralysis of IX X XII cranial nerves left side	10/15	1.5 Neutral 1.2 Inward 0.7 Outward	T Skull base 6x5x5 destruct A blood supply from external carotid artery	1973-75 radiotherapy and microembolization

* Mean value for 500, 1000 and 2000 Hz Rel ISO (1964)

* Patient not in good mental condition

* Discharge and drum perforation

* Radical cavity

ous otitis media the pulse volume in the unaffected ear was $\leq 0.3 \mu\text{l}$. The cases of glomus tumour are listed in Table II.

Fig. 5 illustrates the correlation between the position of the drum and the magnitude of pulse volume in the ear canal in a normal ear. In the neutral position ($P_{\text{ch}}=0$) the volume change is $0.1 \mu\text{l}$. On change in the chamber pressure to $+15 \text{ cm H}_2\text{O}$ resulting in a relative underpressure in the middle ear the drum cupped inwards, and the pulse volume change was somewhat smaller ($0.07 \mu\text{l}$). A corresponding

change in the opposite direction ($-15 \text{ cm H}_2\text{O}$) with a consequent relative overpressure in the middle ear produced exactly the same ($0.07 \mu\text{l}$). The upper part of the curve in Fig. 5 denotes the mean volume displacement of the tympanic membrane in 19 normal ears with different chamber pressures P_{ch} with the eustachian tube closed. The lower part of the figure gives the corresponding pulse volume changes in the ear canal. As can be seen there is practically no difference in the mean volume change within the range of the

s	Postop 1976 ΔV_{pulse} (μl)
rum earing us	0.75 unchanged in diff drum positions
trum earing rugo	Not performed ^a
healing mal - One stop perf rence	Not performed ^a
Discharge 1974 tions rence ged hearing	Not performed ^a
ged hearing nn tus e	Right 0.75 neutral 0.45 inward 0.40 outward Left 0.90 neutral 0.90 inward 0.55 outward
ear drum nn tus ged hearing	0.4 unchanged in diff positions

responding volume change is 1.2 μl . Thus, changes in the position of the drum seem to have a marked effect on the pulse volume change in the presence of a glomus tumour because the pulsations in the tumour probably act directly upon the tympanic membrane.

In serous otitis media the pulse volume change in the ear canal is also large. This is because the fluid in the middle ear transmits the pulsations directly from the mucosal vessels. It is also unaffected by variation of the pressure in the chamber, since the drum remains in the same position irrespective of the pressure changes because the fluid in the middle ear is incompressible.

DISCUSSION

In a normal ear with an intact ear drum the pulse volume change is affected very little by changes in the position of the drum. This means that the pulse volume changes recorded are not transmitted from the middle ear. In one control with a large pulse volume change in the ear canal a skin lesion in the ear canal was probably responsible for the high value (0.38 μl). This change was, however, smaller than in the patient with a glomus tumour (case III) and in whom the pulse volume change was the smallest (0.4 μl) among the cases studied. Testing at different positions of the drum is of considerable diagnostic value as a change in position affects the value considerably in the presence of a glomus tumour, but only little in normals, and not at all in patients with serous otitis media.

The pulse volume change with the drum in the neutral position varied from 0.4 to 3.5 μl in the cases of glomus tumour. The change seems to vary with the size and site of the tumour. At operation, we have found a correlation between the size of the tumour and the pulse volume change. The pulse volume change measured with the drum pushed outward decreased the value recorded in all cases of glomus tumour tested in this way. Furthermore, in these cases a slight inward move-

ent drum positions. Thus, in normal ears a pulse volume change is the result of the pulsations by the outer ear canal and not transmitted from the middle ear mucosa via the ear drum.

Fig. 7 illustrates the effects of drum positions on the pulse volume change in a patient with glomus tumour (case VI). The pulse volume change in neutral position of the drum is 5 μl . When the drum is pushed outward the pulse volume change is reduced to 0.7 μl and when the drum is pushed inward the cor-

No Cases

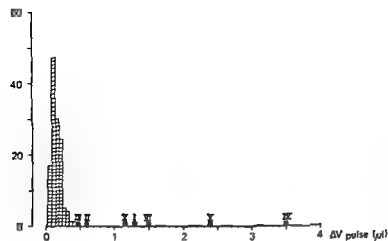


Fig 4 Pulse volume change in normal (filled columns) and in glomus tumour (open columns) Case numbers according to Table II

ment from the neutral position caused a larger pulse volume change than in the neutral position. In this situation the tympanic membrane comes into closer contact with the tumour and thereby increases the pulsations. When the drum was moved further inward the pulse volume changes, except in case III, diminished, probably owing to an effect of changes in the elastic properties of the drum membrane.

In case V postoperative testing showed abnormally large pulse volume changes. The left tympanic membrane exhibited several scars, and it is probable that the pulse volume change is larger in cases with a small volume

of the tympanic cavity and mastoid air cell system and with thin scars, than in a normal ear cavity and mastoid air cell system of ordinary size and with ordinary elasticity of the tympanic membrane. On the right side the pulse volume change was 0.9 μl compared

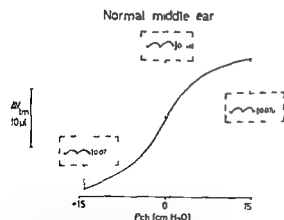


Fig 5 Effects of different drum positions on the pulse volume changes. Neutral position at $P_{ch}=0$ cm H_2O . (Right) the outward bulge (left) the inward cupping of the drum. The pulse volume changes with these positions of the drum are given within the frames.

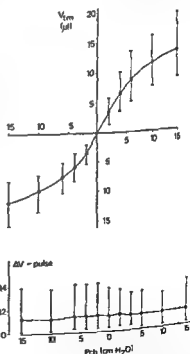
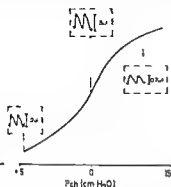


Fig 6 The upper part shows the mean compliance of the tympanic membrane system (19 ears). Neutral position is shown in right upper corner. The lower part shows the pulse volume change (19 ears). The horizontal line is the mean value and the vertical bars the standard deviation.

glomus tumor in the middle ear



Pulse volume changes at different drum positions in case of glomus tumour (case VI). There is a large change in the pulse volume with the drum moved outward.

At a preoperative value of $12 \mu\text{l}$. As the patient also had clinical symptoms of pulse-synchronous tinnitus, there is reason to suspect a recurrence of the tumour. In the lower part of the tympanic membrane a tumour is suspected but the patient is in a poor general condition because of a coexisting malignant disease and no X-ray or operation is tried.

Case VI clearly shows how the method can be used to follow the effect of treatment. The initial pulse volume was $15 \mu\text{l}$ and that observed after radiotherapy was $0.4 \mu\text{l}$, which is almost normal value.

At present we are studying pulse volume changes via the outer ear canal in every case of pulse synchronous tinnitus. If the pulse volume change is abnormal, the pressure chamber technique is used in order to confirm the diagnosis. Then, of course, we use the method when following up the operated cases.

The technique in its present form is not suitable for the general otologic clinic, but comparative studies with the impedance method are in progress, and we hope to present a more simple method in the future. Investigations on other conditions have been commenced in order to find out how they affect the pulse volume change and special

interest is being focused on the question what makes the pulse audible even to a normal person on physical exertion?

ACKNOWLEDGEMENT

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ZUSAMMENFASSUNG

Mit Hilfe eines Volumenstrommessers ist es möglich pulssynchrone volumetrische Veränderungen im äußeren Gehörgang festzustellen. Bei 7 Ohren mit Glomustumoren in der Paukenhöhle und bei 5 mit seröser Otitis media waren diese Veränderungen größer als bei 125 Personen mit normalem Mittelohrbefund. Vorausgesetzt, daß der Patient nicht schluckt, kann das Trommelfell durch Veränderung des Umgebungsdruckes in einer Druckkammer nach medial oder lateral verlagert werden. In sämtlichen untersuchten Fällen mit Glomustumoren wurde ein beträchtlicher Einfluß von diesen Lageveränderungen auf die pulssynchronen volumetrischen Veränderungen beobachtet. Bei Normalpersonen war die Veränderung gering und in den 5 Fällen mit seröser Otitis media blieb sie ganz aus. Die Befunde werden dahingehend gedeutet, daß die Pulsveränderungen bei Normalpersonen hauptsächlich durch die Gefäße im äußeren Gehörgang erzeugt werden.

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ORAL NOREPHEDRINE IN THE TREATMENT OF ACUTE OTITIS MEDIA

Results of a Double blind, Placebo controlled Trial

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Abstract One hundred patients with acute otitis media were treated with oral norephedrine or with placebo for one to two weeks. Symptom cards otoscopy and tympanometry were used for evaluation of the results. There were no differences between the two groups with regard to acute symptoms or healing of the disease. It is concluded that oral decongestants are of no value in the management of acute otitis media.

Impaired tubal function is of significance for the pathophysiology and symptoms of acute otitis media, and especially for the subsequent development of a chronic secretory otitis media. This might be the rationale for the use of intranasal or systemic decongestants in the management of otitis media. Topical vasoconstrictors are difficult to apply in infants and small children. In addition, they have no direct effect on the middle ear and tubal mucosa. For these reasons a test of a systemic decongestant drug in the treatment of acute otitis media seems well motivated. *Norephedrine* was chosen as, in contrast to *ephedrine*, it has little or no effect on heart and CNS when given in ordinary therapeutic doses.

males and 47 females. The mean age was 5.1 years (6 months-40 years). The age distribution is given in Table I.

Sustained release tablets or syrup of phenylamine hydrochloride (*norephedrine*) or of placebo were delivered to the patients in a randomized and double blind basis. The daily dosage of *norephedrine*

Sustained release tablet of 50 mg 1 tablet (50 mg) twice daily for the ages 4-9 years 2 tablets (100 mg) twice daily for the ages ≥ 10 years

Syrup 333 mg per 100 ml 5 ml (16.5 mg) twice daily for the ages 6 months-2 years 7.5 ml (24.75 mg) 3 times daily for the ages 3-5 years 10 ml (33.3 mg) 3 times daily for the ages 6-9 years

performed and score cards collected. Evaluation of these parameters a patient was considered cured the treatment was discontinued and the patient entered the trial. The remaining patients were treated for 2 weeks and were examined again. Treatment was discontinued in all cases after 2 weeks but all pathological changes were corrected by the end of the study.

PATIENTS AND METHODS

The diagnosis of acute otitis media was based on the usual criteria: reddened bulging oedema and immobility of the tympanic membrane. In principle all patients over the age of 6 months entered the trial. The following were not included: (1) 10 children because it was found to be advisable to carry out paracentesis due to extreme pain or excessive fever; (2) 12 children due to inadequate communication with the parents; (3) patients with purulent discharge in the external ear. A total of 110 patients entered the trial and the treatment was completed in 100 having acute otitis media in 144 ears. Of these 53 were

Table I Age distribution of 100 patients with acute otitis media

	No of patients	Active drug
6 months - 2 years	48	25
3 years - 5 years	27	13
6 years - 10 years	18	11
Over 10 years	7	2

• II Mean score for ear-symptoms in patients with acute otitis media, during and after treatment with norephedrine or placebo for days (the lower the score, the milder the pain)

	Norephedrine (51 pats)	Placebo (49 pats)	t test
• a day 2+3 e 0-4)	2.16	1.90	$p > 0.1$
• er of acetylsalicylic tablets used			
• 1+3	1.06	0.80	$p > 0.1$
• ation of treat day 7 based			
• mptom scores			
• additional therapy			
• excellent, 2 good			
• obvious 4 poor)	2.63	2.35	$p > 0.1$
• copy day 7			
• re 1-5)	3.32	3.24	$p > 0.1$
• anometry day 7			
• normal curve			
• flattened curve,			
• negative pressure			
• secretion in the			
• (the ear)	3.00	2.67	$p > 0.1$
• copy day 14			
• re 1-5)	2.83	2.80	$p > 0.1$
• anometry day 14			
• (see supra)	2.59	2.61	$p > 0.1$

in during the period immediately before the actual
tion or had been treated with tubulation of the drum
use of tubal malfunction. Twenty of the 100 patients
given additional therapy, i.e. antibiotics in 13 cases
entesis in 3 and antibiotics and paracentesis in 4. Of
12 were treated with norephedrine and 8 with
•bo

RESULTS

• II shows the symptom scores during the
1 week of treatment and the results of
scopy and tympanometry after one and af-
two weeks' therapy. It is obvious from this
le that norephedrine did not have even the
hlest beneficial effect on otitis media
ptoms. As this total lack of efficacy was
newhat unexpected, some of the remaining
lets and syrup were tested and found to
tain active norephedrine (Lindqvist, 1977)
o proper absorption of the drug was en-
ed by identification of norephedrine in the
ne

Possible side effects were registered in 4
cases. The parents of 2 children, who got
placebo, claimed that the children became
sleepless during treatment. In one child, 2
years old, it is possible that norephedrine ag-
gravated a psychotic behaviour which had
started one week before the trial. The child
was normalized the day after cessation of
treatment. Another 2-year-old child appeared
to be dizzy during norephedrine treatment,
and he got numerous suggestions of the
skin. These symptoms disappeared when
norephedrine therapy was stopped, but this
child was also treated with a fairly large dosis
of acetylsalicylic acid.

DISCUSSION

This trial shows that norephedrine, given in
ordinary therapeutic doses, has no effect on
the acute symptoms or on the healing of acute
otitis media. Although the objective and sen-
sitive parameter—tympanometry—was used,
there was not even the slightest trend, pointing
to any difference between norephedrine and
placebo. Therefore, it seems highly unlikely
that an increased number of patients would
give a different result.

A study (Aschan, 1974) has indicated that
there may exist synergism between a physio-
logical histamine antagonist (a vasoconstric-
tor) and a pharmacological antihistamine com-
pound in the treatment of common cold in-
duced nasal blockage. Thus, it was a possi-
bility that the addition of an antihistamine
compound was also necessary in the treatment
of acute otitis media. However, Sorri et al.
(1977) have recently tested such a combined
preparation (brompheniramine-norephedrine)
in children with prolonged otitis media, and no
significant differences between active and
placebo treatment were disclosed. de Castro
(1974) gave antibiotics to 100 children and anti-
biotics plus a combined preparation (tripoli-
dine hydrochloride and pseudoephedrine
hydrochloride) to another 100. Based on oto-
scopy, 51% in the first and 70% in the second

group were considered to be cured after 10 days. Although this difference is statistically significant, the result of this study is merely indicative, as the trial was not placebo controlled. One large study (Rubenstein, 1967) has seriously questioned the value of oral vasoconstrictor-antihistamine agents in acute otitis media. In conclusion, there seems to be no convincing evidence that peroral vasoconstrictors, either alone or in combination with antihistaminics, are of any value in the treatment of acute otitis media. Topical vasoconstrictors are often used, but the practice is based on pathophysiological theory and not on controlled trials.

Recently Holmquist & Larsson (1976) found that a combined preparation (promethazin and ephedrine) had a normalizing effect on tubal malfunction in adults. Apparently, this is not consistent with our data or with those of Sorn et al (1977), but there may be differences in the pathophysiological basis of the diseases studied, which could account for the discrepancies.

Further controlled studies are required to confirm the significant results of Holmquist & Larsson and to further characterize the group of patients who could conceivably benefit from therapy. Generally speaking, it seems necessary to perform placebo-controlled studies to reconsider the overall management of otitis media, which for many decades has been based on clinical experiences and empirical rather than rational pharmacotherapy (Diamant & Diamant, 1974).

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ZUSAMMENFASSUNG

Hundert Patienten mit akuter Otitis media wurden 1–2 Wochen mit Norephedrin oder Placebo per os behandelt. Otoskopie, Tympanometrie und genaues Symptomenregister dienten zur Grundlage für die Auswertung der Resultate. Es konnten keinerlei Unterschiede zwischen den akuten Symptomen oder im Heilungsverlauf in den beiden Patientengruppen festgestellt werden. Es ergibt sich die Folgerung, daß Norephedrin per os keine Wirkung in der Behandlung der akuten Otitis media hat.

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LABYRINTHINE AND SOMATOSENSORY CONVERGENCE UPON VESTIBULOSPINAL NEURONS

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Abstract In awake cats cells forming the lateral (LVST) medial (MVST) vestibulospinal tracts were identified employing antidromic stimulation of the spinal cord. Axonal responses to bilateral vestibular forelimb hind limb and neck electrical nerve stimulation were analysed. Intracellular recording in the vestibular nuclei was performed via a glass micropipette saturated with Fast Green. Axonal projections were identified in later histological tract identification.

The number of cells projecting to cervical and lumbar regions in the dorsal and ventral division of Deiters' nucleus did not differ significantly. An unexpectedly large number of MVST units was found in the descending nucleus. Some MVST units projected to the lumbar cord but both the medial and descending nuclei projections to the cervical cord were in majority. Almost all spinal projecting vestibular neurons received labyrinthine input and

utilizing multiple pathways. As regards labyrinthine somatosensory integration the two tracts were found to be quite similar. The extent and complexity of labyrinthine somatosensory convergence indicate the importance of vestibular mechanisms upon postural controls also at the level of the vestibular nuclei.

While the vestibular labyrinth is a sensory organ, the vestibular system must be considered as a part of an important motor system, which in conjunction with information derived from other than labyrinthine receptors, is vital to the formation of important reflex responses like the reflexes of balance. The labyrinth influences motor adjustments via the vestibular nuclei (Wilson & Yoshida 1969) which complex (VNC) receives afferent input from the labyrinth and the somatosensory systems (Schwarz et al., 1975) as well as from several prominent descending neuronal systems in-

volved in the regulation of spinal motor activity (Nyberg Hansen, 1966).

There are two major fiber systems projecting from the VNC to the spinal cord: the lateral vestibulospinal tract (LVST), arising in the lateral vestibular nucleus (LVN) and the medial vestibulospinal tract (MVST) originating in essentially the medial (MVN) but also in adjacent parts of the descending (DVN) and lateral (LVN) vestibular nuclei (Nyberg Hansen, 1966, Wilson 1972, Akaike, 1973, Peterson & Coulter, 1977).

Pompeiano & Brodal (1957) demonstrated that the LVST was somatotopically organized and a similar organization has been shown for the MVST by Wilson & Yoshida (1969). In the pigeon, Rabin (1975) pointed out that the association of the labyrinth with limb muscles is insignificant when compared with the association with neck muscles. The importance of neck-vestibular integration in the cat VNC has been emphasized by Rubin et al. (1975).

However, other investigators have indicated that in the cat (Dieter Spiff et al., 1967) and the rabbit (Akaike et al., 1973) stimulation of the labyrinth has a profound effect on motor activity at all levels of the cord. Would one thus expect somatosensory input to the spinal projecting VNC units from all parts of the body equally or would some parts be less repre-

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sented? Somatosensory input to the VNC has been shown to be quite extensive (Allen et al., 1972a, 1972b, Schwarz et al., 1975, Ten Bruggencate et al., 1975) but little information concerning specific labyrinthine and somatosensory input to cord projecting units in cat is available. In the present study, electrophysiological techniques have been employed to investigate the influence of somatosensory and labyrinthine inputs on VNC neurons projecting to the spinal cord.

MATERIALS AND METHODS

The experimental series included 19 adult cats. Surgery was performed under halothane- N_2O-O_2 anaesthesia. Tracheotomy and artificial respiration were routinely employed. End-tidal pCO_2 was monitored continuously with a Beckman Gas Analyzer and kept at 3.5 to 4.5%. A constant body temperature, 37–38°C was maintained throughout each experiment. The halothane administration was stopped prior to recording and the animal immobilized with gallamine triethiodide (Flaxedil). Wounds, pressure points had previously been irritated with Xylocaine, and this local anaesthetic was given repeatedly during the recording. Absence of piloerection and presence of complete miosis indicated absence of pain.

Bipolar silver wires (125 μ m insulated diameter) were positioned on the anterior branches of the ipsilateral (with respect to the recording side) and contralateral vestibular nerves (VN), via an inferior approach to the labyrinth. Identical electrodes were placed on the cochlear nerve within the modiolus and on the facial nerve at its lateral genu. Current spread was controlled by threshold comparison for these 3 electrode pairs, observing evoked eye deviation and neural activity in the VNC. Electrode isolation was secured by paraffin.

The sciatic nerve, the radial nerve and the nerves from C2–C3 to the cleidotrapezoid muscle were dissected bilaterally and mounted on plastic 'rails' containing bipolar stimulating

steel electrodes. No peripheral nerve. Manual stimulation (touch, rotation of pressure to muscles etc.) of different parts gave additional information concerning types of somatosensory input to the cells.

The C2–C4 segments as well as the L3 segments were exposed through a dorsal laminectomy. An insulated silver wire (125 μ m) exposed at the tip was placed on the surface of the cervical cord near the midline at the C3–C4 level. A second wire was placed under the cord and placed on the ventral surface. A similar procedure was performed at the L3–L4 level, thus permitting bipolar stimulation of the different spinal segments.

Following posterior craniotomy the brain was placed in a David Kopf stereotaxic frame. A recording glass micropipette (NaCl, 1–3M Ω) saturated with Fast (FCF) was introduced obliquely into the brain stem above the obex. At the bottom of the track dye was iontophoretically ejected to mark the location of tip (Thomas & Whitlock, 1965). The VNC was reached without penetrating the cerebellum. The placement of the electrode in the VNC was based upon stereotaxic coordinates and the physiological technique of Shimazu & Precht (1965).

The electrical nerve stimulus consisted of square waves (0.1 msec duration) with a frequency of 1.5 times threshold (evoked eye deviation and muscle twitches respectively). The frequency was usually 1 stim/sec.

Unitary spike activity and field potentials were displayed on the oscilloscope after conventional amplification (bandwidth 1 Hz – 300 Hz for field potentials, 100 Hz – 1 kHz for spike potentials). Additional analysis of unitary activity was made with a laboratory computer (PDP 8E, peristimulus time histograms, adjustable bin widths for 100 bins histogram).

Units in the VNC projecting to the cervical and lumbar levels were identified by antidromic stimulation. It was of prime importance that only certain catenae were

Table I Projection of vestibulospinal units

Leus	To cervical cord (C cells)	To lumbar cord (L cells)
✓	31	32
LVN	19	15
- LVN	12	17
H	41	7
N	15	1
- al	87	40

tested for antidromic activation (Kelly et al., 1975, Fuller & Schlag, 1976)

Once a unit was identified as a projecting unit its spontaneous activity (if any) was recorded. The neuron was then subjected to a battery of stimuli, the response to bilateral subular, neck, radial and sciatic nerve stimulation was investigated as was the response to manual somatosensory manipulation.

At the end of the recording session the cat was killed with a lethal dose of Nembutal and perfused with 10% formaldehyde. The brain stem was removed, frozen, and serial transverse sections were cut at 100 μ m. The dye marks could be identified in these sections which were stained by the Kluver Barrera method. The various nuclei were delineated according to Berman (1968). Cells which were found to lie on or near nuclear boundaries (especially within LVN) were rejected in order to minimize the inclusion of any LVST projecting units from other than LVN and of MVST units from other than MVN or DVN.

RESULTS

A total of 127 neurons projecting to the spinal cord were included. Based upon the results of various investigators (see Pompeiano 1972 for a review) units located clearly within the borders of the LVN sending axons to the spinal cord were considered part of the LVST and neurons lying clearly within the boundaries of the MVN and DVN were correspondingly included into the MVST. As shown in

Table I the largest part of projecting units was found in the LVN. The antidromic spikes recorded in the LVN had latencies ranging from 0.8 to 1.7 msec for cervical stimulation and 2.4 to 5.3 msec for lumbar stimulation.

While cervical cord projecting units (C cells) predominated overall, lumbar cord projecting units (L cells) were most frequent in the DLVN. There was a small difference in the numbers of C and L cells in VLVN and DLVN (ventral and dorsal parts of the LVN).

MVST units fired their antidromic spikes with latency range of 0.5–3 msec. In both the MVN and DVN the projection to the cervical cord far outnumbered the projection to the lumbar cord—an indication of the importance of this tract in orientation of the upper body. As noted in Table I an interesting finding is the relatively large number of spinal projecting neurons in the DVN. There also appeared to exist direct pathways, although few, between MVST units and the lumbar cord.

The superior vestibular nucleus (SVN) completely lacked neurons projecting to the cord judged from a sample of 203 units.

Labyrinthine Input to Spinal Projecting Units

As indicated in Table II some units projecting to the spinal cord received no labyrinthine input. However, the vast majority (85%, $n=127$) received input from labyrinthine receptors. The units in the MVN and in the DVN, showed predominantly bilateral labyrinthine influence (54%, $n=48$ for MVN, 56%, $n=16$ for DVN). The projecting LVN units, however, manifested a preference for ipsilateral labyrinthine input (68%, $n=63$). No vestibulospinal unit with only contralateral labyrinthine input was ever encountered. The MVST units of the DVN were found to contain the largest percentage (25%, $n=16$) of units not responding to labyrinthine stimulation, the small sample, however, makes conclusive statements impossible.

Neurons influenced by ipsilateral labyrinthine stimulus were analysed as regards their

Table 2 *Labyrinthine and somatosensory input to vestibulospinal VNC units*

Abbreviations DVN=descending vestibular nucleus LVN=lateral vestibular nucleus LVST lateral vestibular tract MVN=medial vestibular nucleus MVST=medial vestibulospinal tract SI=somatosensory input VNC=vestibular nuclear complex

	MVN Units		DVN Units		LVN Units		Total
	SI	no SI	SI	no SI	SI	no SI	
From ipsilateral labyrinth only	6	8	2	1	19	24	60
From contralateral labyrinth only	0	0	0	0	0	0	0
From both labyrinths	23	3	7	2	12	2	49
No labyrinthine input	8	0	4	0	6	0	18
Total	37	11	13	3	37	26	177

response latencies. Due to the difficulty of determining exactly where an inhibition begins, bin widths of 0.5 msec were employed (Fig. 1) for inhibited units. These units often also showed an excitatory response which is included in the excitatory response data. In order to separate monosynaptic from disynaptic and polysynaptic excitatory responses, latencies were examined using bin widths of 0.1 msec (Fig. 1A). Employing the monosynaptic latency grouping of Wilson et al. (1967), all units discharging within the latency range of 8–15 msec were classified as monosynaptic (i.e. all spikes that occurred 0–0.6 msec after the start of the N_1 potential). Units with a response latency of 1.6 to 2.2 msec were classified as disynaptic (Ito et al., 1969). Those with latencies >2.2 msec were considered polysynaptic.

Monosynaptic units were found for both LVST and MVST systems. The LVST units appeared to fall into three major latency populations (Fig. 1A). One population showed monosynaptic responses centered around 1.1 msec, a second around 2.9 msec while a third one was concentrated around 4 msec. The MVST units appeared to contain only two latency populations. The early monosynaptic one coincided with that of the LVST units and the second population centered around 2.5 msec. No spinal projecting units were encountered answering to ipsilateral vestibular stimulus with a disynaptic latency.

An interesting finding was the relatively

large number of units inhibited by labyrinthine input (Fig. 1B). The earliest inhibitory response was 3 msec for both LVST and MVST units. As was seen for excitatory responses, the inhibitory ones fell into distinct latency groups. LVST neurons centered around

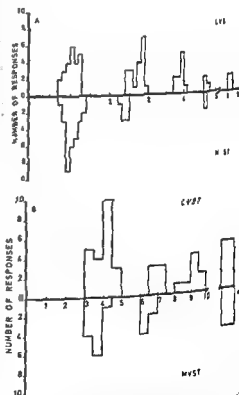


Fig. 1. Latency histograms for ipsilateral labyrinthine input to vestibulospinal cells. (A) Excitatory LVST responses are indicated above the x-axis. The MVST below the x-axis. Bin widths of 0.1 msec were employed up to 5 msec. Inhibitory responses are similarly displayed. Bin widths of 0.5 msec were employed up to 10 msec.

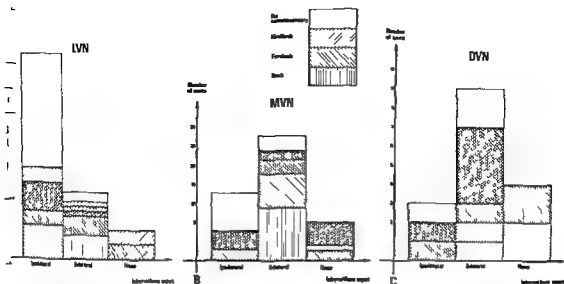


Fig. 2 The distribution of labyrinthine and somatosensory input to the vestibulospinal cells. The units were divided into three groups based on labyrinthine input: ipsilateral, bilateral and no labyrinthine input. Units receiving solely contralateral labyrinthine input were absent. For each column

the somatosensory input patterns and proportions are indicated. (A) Units in the lateral vestibular nucleus (LVN); (B) Units in the medial vestibular nucleus (MVN); (C) Units in the descending vestibular nucleus (DVN).

and 9 msec while in the MVST population latencies gathered around 4 and 6.5 msec.

Somatosensory Input to Vestibulospinal Units

(a) LVST

Somatosensory input was noted in 58% ($n=3$) of the LVST units and 49% displayed somatosensory and labyrinthine convergence. To facilitate analysis of this convergence, LVST units were divided into four categories based upon the pattern of labyrinthine input demonstrated in Table II. Most LVST units received an ipsilateral labyrinthine input. Of these, 56% ($n=43$) received no somatosensory input while the remainders had somatosensory receptive fields most frequently engaging forelimb regions (27%, $n=19$) or larger body areas (neck, forelimb and hindlimb, 32%, $n=19$) (Fig. 2A). Twenty-two per cent of the LVST cells received bilateral labyrinthine input and 86% ($n=14$) of these responded to peripheral nerve stimulus and/or manual somatosensory activation.

Of the 63 LVST units 6 did not respond to labyrinthine input but could all be demonstrated to receive somatosensory input. Thus all LVST units received labyrinthine and/or somatosensory input. The LVN neuron in Fig. 3 belongs to the group influenced by both labyrinths but with no proven somatosensory input.

(b) MVST

MVN 77% ($n=48$) of the units responded to neck or limb nerve stimulation and in 60% a labyrinthine-somatosensory convergence was demonstrated. Of all MVN units, 23% responded to labyrinthine input exclusively and 17% were affected only by somatosensory input. No MVN units were devoid of afferent input (Table II). Activation from both labyrinths occurred for 53% of MVN cells and of these neurons somatosensory activation was noted in 88% ($n=26$). The most common somatosensory response (48%, $n=23$) was seen following neck nerve stimulation (Fig. 2B). Fig. 3 (D-F) illustrates a convergence

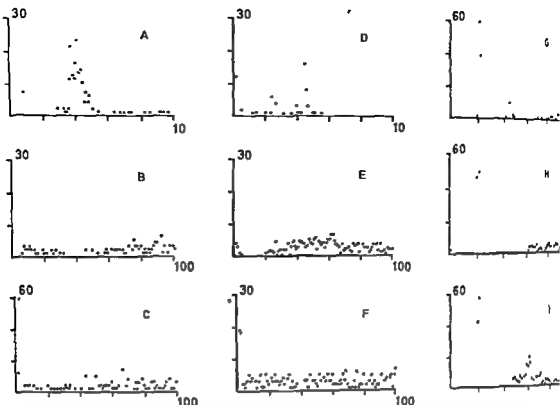


Fig 3 Response latencies of three representative spinal projecting vestibular neurons (A-C) LVST unit responding only to vestibular stimulation with ipsilateral excitation (A) and contralateral inhibition (B) (C) shows resting charge (D-F) MVST unit in the medial vestibular nucleus showing labyrinthine/neck convergence with an excitatory response to ipsilateral labyrinth (D) and inhibition

to ipsilateral neck stimulation (E) (F) is spontaneous activity (G-I) MVST unit in the descending vestibular nucleus receiving convergent input from labyrinthine/neck (G) is the excitatory response to ipsilateral labyrinthine stimulation (H) the inhibitory influence of contralateral neck stimulation while an ipsilateral one produces the same type of response but with a shorter latency

MVN neuron being excited by ipsilateral labyrinthine stimulation and inhibited by ipsilateral (C_2 - C_3) neck nerve stimulation

As noted above for LVST units, no MVST units were recorded which responded solely to contralateral vestibular stimulus. An ipsilateral input was seen in 29%, but in contrast with data in the "bilateral" group, the majority (57%, $n=14$) received no somatosensory input (Table II, Fig 2B). When labyrinthine-somatosensory convergence was observed the somatosensory receptive fields usually included neck and limb areas in both body halves (Fig 2B). Unlike the LVST, no unit in MVN or DVN responded to only sciatic nerve stimulation.

DVN 81% ($n=16$) had somatosensory in-

put, 56% showed labyrinth-somatosensory convergence. Twenty-five per cent of the units were influenced by somatosensory input. No MVST units were free of afferent input (Table II; Fig 2C). The largest group was activated from both labyrinths (56%, $n=11$). In this group 7 units were convergent units, also received somatosensory input. The common somatosensory response followed neck, forelimb and hindlimb stimuli (Fig 2C). No units had contralateral labyrinthine input only. Fig 3 (G-I) shows MVST unit convergence of ipsilateral labyrinthine input responding monosynaptically with bilateral neck input. Due to small sample of units, comments regarding representative types of somatosensory input patterns are impossible.

DISCUSSION

ny anatomical and physiological studies e confirmed the existence of two m- vestibulospinal projection systems. The ST is acknowledged to arise almost exclu- ly from Deiters' nucleus (LVN) while the ST has been shown to have its origin in the VN and adjacent DVN and perhaps LVN (Nygberg Hansen 1966, Wilson, 1972, Akaike, 1973, Peterson & Coulter, 1977). By restricting analysis to those units which lie clearly within the boundaries of each nucleus, very few MVN or DVN units included here are likely to project via the LVST. Similarly, the likelihood of LVN units examined projecting to the MVST is minimized.

The present study has shown that vestibular units often receive different patterns of labyrinth-somatosensory input depending upon the nucleus of origin.

The basic assumption has previously been made that the contribution of VLVN and DLVN units to the LVST did not differ significantly (Wilson 1972). The present data based on a comparable number of electrode tracks and analysed neurons in the two nuclear subpartitions support this observation (Table 1). The rejection to spinal levels is however different, a finding also noted by Wilson et al (1967).

It would appear that there is a larger projection of the DLVN to the hindlimb control areas of the cord. The VLVN appears to show greater projection to upper body areas—neck and forelimbs—but also contains many hindlimb projecting units. The projections to the cervical and lumbar cord are separate. However, Abzug et al (1973), have found many LVN units influencing both lower cervical and lumbosacral regions of the spinal cord. If branches of LVST axons act on both forelimb and hindlimb motoneurons they will impose a degree of forelimb-hindlimb coordination in addition to the forelimb-hindlimb reflexes that are present. In this study LVST branching units were not studied.

Inhibitory responses to labyrinth input were commonly noted for LVN cells. Such responses were absent in the study by Wilson & Fessel (1972). It is possible that the presence of an intact cerebellum was responsible for the inhibitory responses (Ryu & McCabe, 1976).

Slightly more than half of the LVST units (37/63) had a somatosensory input and almost one half (31/63) showed labyrinth/somatosensory convergence. The LVST regulates motor activity through direct and polysynaptic connections (Hongo et al, 1975) with alpha and gamma motoneurons and by modulating segmental reflexes by means of terminations on interneurons in their pathway (see Grillner, 1972, for a review). Such a system is highly dependent on feedback information which is illustrated by the large percentage of somatosensory afferents seen in this study.

Certain LVST units can also be activated by labyrinthine impulses. Furthermore, they are under the control of cerebellum and of impulses of somatic and other origins (Wilson, 1972) making coordination of this complex system possible. LVST units are subsequently not simple relay units for labyrinthine commands to the spinal cord motoneurons but they receive somatosensory information for feedback (to spinal cord) and feedforward (to higher centers of CNS) processes, as well as labyrinth-somatosensory convergence in order to integrate information from different modalities.

The majority of MVST units in the present study were antidromically activated by cervical cord stimulation. Stimulation of the MVN and DVN may influence the lumbar segments of the spinal cord bilaterally (Pompeiano, 1972). As a result, two main pathways have been described through which vestibular impulses may reach the lumbar cord: (1) via vestibuloreticular fibers from the MVN and DVN (Ladplig & Brodal, 1968) relayed to the cord via bilateral reticulospinal fibers which can reach the lower spinal segments (Nygberg Hansen 1966), (2) via descending propriospinal

nal fibers interconnecting the spinal enlargements (Giovannelli & Kuypers 1969)

However, the above two pathways act on the lower cord via synaptic pathways whereas in the present study some of the MVST units appeared to project directly to the lumbar cord. Most of these MVST units displayed extremely rapid labyrinthine responses (3 msec). In fact, more than half of the neurons activated by ipsilateral labyrinth stimulation showed monosynaptic discharge. Wilson et al (1968) also found when employing decerebellate cats that among cells projecting to the MVST, a greater percentage received monosynaptic input than any other group of cells studied in the MVN.

The MVST system is considered by many authors to reach no further caudally than to the mid thoracic cord (Brodal et al, 1962, see however, Akaike, 1973) and to have a strong direct input from labyrinthine receptors (Wilson et al, 1967). Except for the minor contingent of MVST units projecting to the upper cord, the present findings are in agreement with these opinions. These findings together with the data that the cervicothoracic component of the LVST is relatively more subject to labyrinthine control than the lumbosacral component (see Wilson, 1972 for a review) explain the observations that the motor activity of the cervicothoracic cord is more influenced by the labyrinth than motor activities of the lumbosacral cord. However, it must be emphasized that the MVST action on the cervicothoracic cord is particularly directed towards neck motoneurons rather than forelimb motoneurons.

Most of the MVST units showed labyrinth-somatosensory convergence with somatosensory receptive fields frequency in upper parts of the body. Yet, many of these convergent units also responded to hindlimb input. Mori & Matsumoto (1972) have demonstrated the existence of a system within the spinal cord transmitting input from the neck to forelimb motoneurons and modulating the excitability of the flexor reflex. It is quite likely that such

a system also exists for neck-hindlimb and forelimb-hindlimb coordination since the limb adjustments are part of the overall postural changes when the balance of the animal is upset (Roberts, 1967).

As noted for the LVST the MVST units, both DVN and MVN, received inhibitory input. A grouping into latency populations for both excitatory and inhibitory input to the units was observed which may imply two pathway input systems to the VNC.

It can be concluded that the LVST and MVST units show a difference with regard not only their site of origin but also concerning the response characteristics to multiaxial stimuli.

ZUSAMMENFASSUNG

Bei wachen Katzen wurden die Neuronen der lateralen (LVST) und medialen (MVST) Tractus vestibularis durch antidrome Stimulation des N. vestibularis

wurden analysiert. Eine extrazelluläre Kapselierung Glasmikropipetten gefüllt mit Fast green wurden in Vestibularkernen durchgeführt um eine spätere histologische Identifikation möglich zu machen. Es gab keinen signifikanten Unterschied zwischen dem dorsalen und ventralen Teil von Deiters' Kern in Bezug auf die Anzahl der Neuronen, welche Reizantworten in der Hals- und Lendenregion auslösten. Eine unerwartet große Anzahl von MVST Neuronen wurde im kaudalen Kern gefunden. Einige MVST Neuronen lösten im Lendenmark Reizantworten aus, aber sowohl der mediale als auch der laterale Kern lösten überwiegend Reizantworten in der Halsregion des Rückenmarks aus. Beinahe alle Vestibularkerne, die im Rückenmark Reizantworten auslösten, erhielten Innenohreinfluß und mehr als die Hälfte waren sensiblen für den sensorischen Einfluß. Die Neuronen konnten auf Grund unterschiedlicher erregender und hemmender Antworten des Labyrinths in verschiedene Gruppen eingeteilt werden, die auf mehrfache Wege schließbar waren. Die Integration der Labyrinthbahnen und der somatosensory Bahnen betrifft erwachsenen Menschen ständig gleichwertig. Der Umfang und die Komplexität der Labyrinth-somatosensory Konvergenz erwachsenen Menschen hat Bedeutung für die Ruckkopplungsmechanismen für die Raumlagekontrolle auch auf dem Niveau der Vestibularkerne.

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It can be concluded that the LVST and MVST units show a difference with respect not only their site of origin but also concerning the response characteristics to multiple stimuli.

ZUSAMMENFASSUNG

Bei wachen Katzen wurden die Neuronen die

in der Hals- und Brustregion des Vestibulärnervs auf beiden Seiten der Nerven der Vorder- und Hinterbeine sowie des Kopfes wurden analysiert. Eine extrazelluläre Registrierung mit Glasmikropipetten gefüllt mit Fast green wurde in den Vestibulärkernen durchgeführt um eine spätere histologische Identifikation möglich zu machen. Es gab einen signifikanten Unterschied zwischen dem dorsal-lateralen und ventralen Teil von Deiters' Kern in Bezug auf die Reizantworten der Neuronen, welche Reizantworten in der Hals- und Brustregion auslösten. Eine unerwartet große Anzahl von MVST-Neuronen wurde im kaudalen Kern gefunden. Einige MVST-Neuronen lösten im Lendruckmark Reizantworten aus, aber sowohl der mediale als auch der laterale Kern lösten überwiegend Reizantworten in der Brust- und Halsregion des Rückenmarks aus. Beinahe alle Vestibulärkerne

zeigten eine Konvergenz von Reizantworten auf labyrinthinäre und somatosensorische Reize. Die Integration der Labyrinthbahnen und der somatosensorischen Bahnen betrifft erwiesenermaßen beide Seiten des Körpers gleichwertig. Der Umfang und die Bedeutung der Labyrinth-somatosensorischen Konvergenz für die Bedeutung von Rückkopplungsmechanismen für die Raumlagekontrolle auch auf dem Niveau der Vestibulärkerne.

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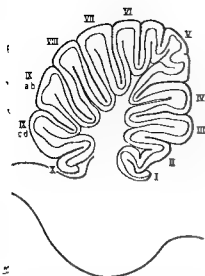


Fig. 1. Diagram of a sagittal section of the cerebellar cortex of a pigeon. Lobules are numbered according to Larsell (1967). The stippled area indicates the region exposed with microelectrodes.

maintained by a constant gas stream through a tracheostoma and a cannulation of the abdominal air sack and paralysed with Flaxedil.

The head was clamped in a stereotaxic frame (a modification of Karten & Hodossy, 1967) device to permit a more rigid fixation in place which was mounted on a rotation table with the center of the interaural axis coincident with the center of rotation. Position of the lateral semicircular canals was adjusted to be approximately coplanar with the rotation plane, it could, however, be tilted laterally (in relation) out of this plane by up to 40° to either side. The body was placed into a snugly fitting plaster of Paris cast mounted on a metal bar which could be rotated about three axes (roll, pitch and yaw) with respect to the head, the corresponding joints being located just under the pigeon's head. The yaw axis joint was attached to a potentiometer so that neck joint position could be monitored. During rotation of the table, body position was always fixed with respect to the head, as was the tilt angle of the head with respect to the table. The axis of the table was directly mounted to a servo-controlled torque motor driven by a function

generator to provide sinusoidal rotations of 0.25 or 0.5 Hz with maximal angular velocities of ca $60^\circ/\text{sec}$.

The bone overlying cerebellar lobules VI, VII and VIII (Larsell, 1967) was removed and the dura mater incised to permit systematic scanning of the underlying cerebellar cortex (Fig. 1) for neurons responding to rotation in the horizontal canal plane. The halothane anaesthesia was discontinued prior to recording, leaving the animal under the analgesic action of N_2O and Ketamine. Extracellular neuronal activity was recorded with glass micropipettes filled with 2 M NaCl (impedance 1–5 M Ω at 1 kHz). These electrodes were driven vertically (with respect to horizontal canal plane) through the cortex, the cortical layers being roughly identified by background activity patterns. During penetration, the preparation was subjected to manual rotation in the horizontal plane until responsive neurons were isolated. Each of these neurons was further investigated with controlled horizontal rotatory stimuli with the head held horizontally or at a lateral angle of up to 30° . Responses to lateral tilting movements (about the

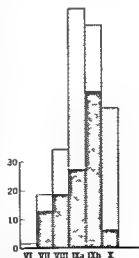


Fig. 2. This histogram indicates the relative numbers of cells in lobules VI through X which responded to vestibular (light stippling) or both vestibular and neck (dark stippling) stimuli. Lobule IXa" includes IXa and b. IXb" includes IXc and d according to Larsell (1967).

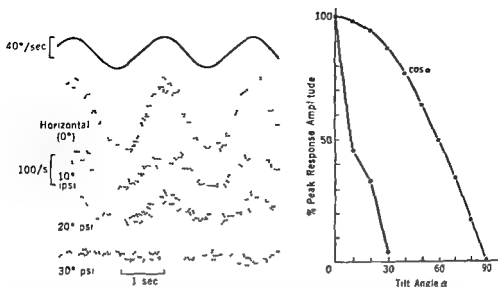


Fig 3 Histograms represent the responses of a neuron to 16 identical horizontal rotations at four different tilt angles (0°, 10°, 20°, and 30° tilt with ipsilateral side down). Head velocity is shown in the top trace. The diagram on the

right is a plot of the response amplitude of this cell as a function of tilt angle. The theoretical cosine response is also plotted for comparison.

roll axis) were also noted. Then the body was manually moved about each of the three available neck rotation axes in search for neuronal responses.

Conventional *in vivo* recording techniques with a bandwidth of 1 Hz to 10 kHz were used. Spikes of identified neurons were filtered at 300 Hz and transformed, by means of a window discriminator, into standard pulses. These were counted by computer to produce stimulus time histograms for a number of identical rotatory sinusoidal stimulation cycles (usually 16). Standard pulses were also used to compose instantaneous diagrams of frequency versus time for manual stimuli which were not suitable for averaging techniques.

During each experiment a number of electrode tips were left *in situ* so that tip positions in histological slides could be compared with the same positions determined electrophysiologically. For this purpose the brains were perfused transcardially under halothane anaesthesia with a fixative. The cerebella cut at 90 μ m on a freezing microtome and stained with cresyl violet. Since there was a good agreement between these techniques, the histological location for each investigated neuron could

be extrapolated from electrophysiological data for all other recording locations.

RESULTS

Since it had been shown in a previous study that neck and labyrinthine impulses converge in lobules VII, VIII, IX and X of the avian cerebellum (Schwarz et al, in press), neurons were sampled throughout the region illustrated in Fig 1. This distribution of convergence was confirmed in the present study, emphasizing natural stimuli for both afferent systems. Fig 2 illustrates the distribution of vestibular (left)

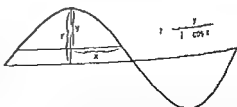


Fig 4 This diagram explains the equation used to calculate the response amplitude when only a portion of the sine wave was present. r = Theoretical response amplitude. $x = 1/2$ response duration expressed as an angle. y = observed response amplitude.

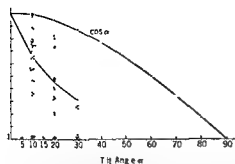


Fig. 5. Diagram of the response amplitude at different angles expressed as a percentage of the peak response of a population of cerebellar cells. The predicted cosine function is plotted for comparison. The solid line contains the mean response amplitudes at tilt angles of 10° and 30°.

plung) and vestibulo cervical (dark strip) neuronal responses in these lobules. This diagram includes all vestibular responses, but only those classified as horizontal canal responses and described in detail below, however, neck input is only included when it coincides with vestibular input. It is remarkable that very few neck afferents appear to be directed to the nodulus (5%), whereas most are seen in the uvula (68%). This matches the pattern seen in the cat (Schwarz & Milne, 1976). Direct vestibular fibers are directed primarily to the ventral portion of the uvula and the nodulus (Whitlock, 1952; Schwarz & Schwarz, in preparation).

The histogram of Fig. 2 is biased towards semicircular canal responses since only cells responding to horizontal rotation were searched for detailed study. As we wished to select units specifically responding to horizontal canal stimulation, criteria for this put had to be adopted. We believed initially that the following three criteria would select the desired neurons:

- 1) the cell should not respond to lateral tilting movements
- 2) the phase angle between stimulus in the horizontal plane and response should indicate a reasonable correlation between unitary activity and head velocity (cf. Goldberg & Fer-

nandez, 1971; Lufschitz, 1973; Blanks et al., 1975).

3) when the pigeon's head was tilted about the roll axis the amplitude of the neuron's response to horizontal rotation should diminish according to a cosine function (cf. Blanks et al., 1975).

While there were many neurons to satisfy the first two criteria, not one was found acceptable according to the third one.

An example is given in Fig. 3. This neuron exhibited no detectable change in background activity with angular acceleration about the roll axis. The velocity of horizontal rotation is shown on top. The four histograms below show responses to horizontal rotation when the head was positioned horizontally and tilted by 10°, 20° and 30° towards the ipsilateral side. A similar response series was seen with contralateral tilt. It is evident that modulation of background activity is approximately in phase with head velocity and that response ampli-

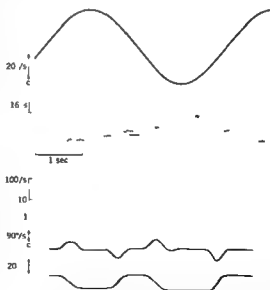


Fig. 6. Responses of a neuron to head and neck rotation. Top trace=head velocity; second trace=histogram of response to sixteen identical head rotations; third trace=instantaneous firing frequency in response to rotation of the neck; fourth and fifth traces represent neck velocity and position respectively. *i*=Ipsilateral; *c*=contralateral.

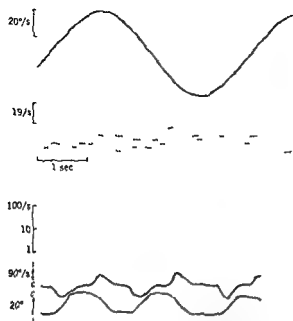


Fig 7 Responses of a neuron to head and neck rotation. Top trace=head velocity, second trace=histogram of response to sixteen identical head rotations, third trace=instantaneous firing frequency in response to rotation of the neck, fourth and fifth traces represent neck velocity and position respectively.

decline with tilt about the roll axis. However, the amount of response decline is far greater than predicted by the cosine function. For example, 87% of the greatest response amplitude should still be present with a 30° tilt according to that function, whereas no response is present in the bottom histogram. In the diagram to the right the response decline seen is compared with that predicted by the cosine relationship. Evidently this cell is much more specifically tuned to the horizontal plane than a primary horizontal canal cell should be.

The cell of Fig 3 was not typical for our population of 96 neurons receiving horizontal canal input (so classified in spite of criterion 3) since its response represented an almost complete sinusoid. For most cells only a segment of the sinewave was present in the response (e.g. Fig 8). As Fig 4 shows, the amplitude and duration of such a segment can be used to examine the validity of the cosine relationship for cerebellar neurons responding to semicircular canal input. The peak of the sine

wave obtainable by regression from a histogram containing only a sinewave segment can be obtained using the relationship to r of Fig 4, where r is peak amplitude, y is observed amplitude and x half of the period duration expressed as an angle.

Peak amplitudes of response sinusoids obtained for many units according to Fig. 4 for different tilt angles are summarized in Fig. 5 where they are expressed as percentages of the maximal amplitude obtained in the horizontal plane. It is clear that all amplitudes at angles between 5° and 30° are small; they should be according to the cosine relationship indicated in this graph. A solid line connects the means at 10°, 20° and 30°, illustrating a considerably greater planificity for the cerebellar neuron population than for known primary afferents.

Since the horizontal canal plane is more precisely represented in the cerebellum than indicated by our third criterion, we accept "horizontal canal cells" all those cells satisfying the first two criteria and exhibiting a maximal response amplitude in the horizontal plane. The vast majority of these cells were recorded from the granular layer; only a few from the neighbourhood of the kinesthetic cell (PC) layer (in our laboratory this layer cannot be identified without antidromic stimulation since interneurons are expected to produce complex spikes). Responsive cells were found in the granular layer where extracellular spikes had long durations (5–40 msec) (cf. Linas & Dichgans 1969 for an interpretation) and fired at a low background frequency (below 2 Hz). Of 41 horizontal canal cells, 41 responded to rotation as well. All of these neurons responded to neck movement about the y-axis with only 3 giving clear although weak responses to roll movements as well. All changed their firing rate with pitch movements.

Examples are given in Figs 6 to 8. The horizontal canal cell of Fig 6 exhibits a tonic response to ipsilateral movements.

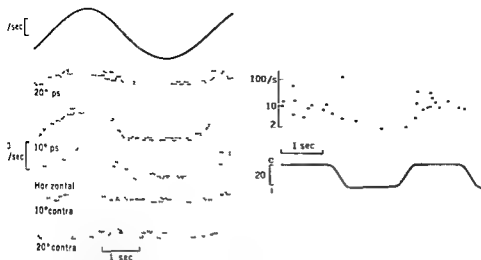


Fig. 8. Histograms on the left show responses to horizontal rotation at five different tilt angles (upper trace = velocity). Diagram on the right illustrates the re-

sponse of this cell to neck rotation (lower trace = neck position).

with respect to the head, thus signalling head position. The vestibular response encoded head velocity towards the contralateral side. Since contralateral body movements can modulate the cell's activity due to vestibular stimulation acts upon a background of neck position. Few such purely tonic responses were seen. The neuron of Fig. 7 responds in a purely phasic fashion to ipsilateral neck movements in yaw, signalling a change in neck position. This phasic neck response can easily override the vestibular signal for head velocity towards the contralateral side when the pigeon moves its head under natural conditions. This type of neuron may be related to vestibular nuclei cells, which have been shown to code both neck and head velocity (Rubin et al., 1975). Unfortunately the usual analysis techniques available for neck responses in this study did not permit a decision as to how well the firing pattern of phasic responses corresponded to velocity.

Responses of all 41 neurons to yaw neck movements were directionally specific, neck movements towards ipsilateral being excitatory and towards contralateral inhibitory or vice versa. The directional specificity encoded in neck responses did not, however, match that

displayed in the horizontal canal responses in a systematic fashion about as many activations in yaw towards ipsilateral converged with canal activation due to ipsilateral head rotation as with activations due to rotations towards the contralateral side. That is to say, the chances that information from the neck is cancelled by the labyrinth due to subtraction when the pigeon moves its head towards one side are about as great as those of the generation of a stronger signal due to summation of both inputs.

Fig. 8 shows an aberration from the normal response pattern seen only rarely in this study (4 cells). The maximal amplitude to rotation is seen when the head is tilted by 10° ipsilaterally rather than in the horizontal plane. All other neurons recorded during this experiment exhibited the usual maximum in the horizontal plane. It will be recognized that in all other aspects this neuron reacted like horizontal canal cells, encoding head velocity and not responding to tilt movements in roll. The neck response is an activation during a contralateral yaw deviation.

It is noteworthy that the neuron population described thus far represents a carefully biased sample. Many neurons responding to tilt about

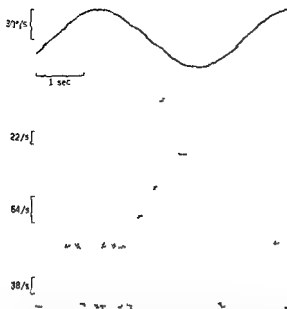


Fig 9 Responses of four different cells to horizontal sinusoidal rotation. Histograms represent responses to sixteen identical stimuli. Head velocity is illustrated in the top trace.

the roll axis were not analysed in detail. It is interesting to note, however, that all these neurons responded to pitching or rolling neck movements with very few changing their activity during yaw movements, provided neck input was present at all.

Many neuronal responses to horizontal head rotation did not justify classification into the horizontal canal group, although the horizontal canals may well be involved in their generation. Examples for four different cells are given in Fig 9. The first histogram has two peaks of excitation, one in phase with head velocity and one leading (or lagging) head velocity by ca. 90° . A convergence of a velocity and position coding element could account for this pattern (e.g. convergence of a primary canal afferent with an indirect canal afferent after integration). The response illustrated in the second histogram might (or might not) be interpreted as coding position, which could of course also be caused by otolithic input. A curious trapezoidally shaped pattern, such as in the third histogram, was seen quite often. This response is not a clipped segment of a sinusoid, since smaller re-

sponses caused by lateral tilt had the trapezoidal shape. Finally, it should be forgotten that in almost all studies of the literature a number of entirely uninterpretable response patterns can be seen, such as in the last histogram.

DISCUSSION

Two lines of evidence emerging from the reported above as well as from earlier (Schwarz & Tomlinson 1977) indicate that the cerebellar cortex receives rather specific information about movement direction signalling horizontal head rotation is more precise than available from known first-order lateral semicircular canal afferents and that afferents specifically converge with somatosensory afferents informing about neck movements in a corresponding plane.

No mechanism is known that can give greater precision for vestibular signalling the plane of horizontal head rotation than given by a cosine function. This function describes the amount of canal stimulation in any given plane of rotation and has been shown to be an accurate description of few semicircular canal afferents in the rat (Blanks et al 1975). According to this, neuronal responses to horizontal rotation should change very little if the rotation is tilted out of its plane by small angles and the amplitudes should drop rapidly to zero as the tilt angle approaches 90° . The corresponding null point at 90° can be used to identify a given unit in the vestibular nerve (Estes et al 1975; Blanks & 1976). Null point or better null area determination would be a very insensitive classification technique in the avian cerebellum where the canal plane is encoded in firing terms much more precisely. Contrasting circuitry in the brain is probably required to achieve this precision. For example, convergence of inhibitory vertical and horizontal canal input could conceivably reduce the observed effect. This pro-

d be difficult to reconcile with the fact very little convergence between electrically stimulated canals could be seen in the dorsal cerebellar cortex (Wilson et al., 1974, and in preparation). Nevertheless, the possibility of canal interaction producing increased plane specificity has to be investigated. Of course other plane sharpening circuitry is conceivable. It is, finally, still possible (although not probable) that plane specificity is greater than given by a cosine function. This is available in primary afferents. Threshold measurements to angular acceleration for primary canal afferents in the dogfish indicated threshold differences as long as the lateral canal was within 15° – 20° of its plane (Lowenstein & Sand, 1940). This "latitude" (Lowenstein, 1974) was greater for vertical canals than for the horizontal canal and these ranges thresholds rise steeply. These observations might suggest a deviation from the cosine law, they do, however, not show such unexpected behaviour of primary canal afferents because

- 1) one cosine function can only represent one plane. If the canal is bent to represent several planes (as vertical canals in many species do) the sum of several cosine functions should describe firing patterns.
 - 2) threshold measurements are poor parameters to assess response magnitude since observed thresholds for semicircular canal afferents always depend upon the sensitivity of recording equipment employed.
- It can certainly not be assumed that the few first order neurons tested as to this question represent the total range of the semicircular canal afferent population and it is interesting to note in this context that efferent fibers are supposed to be inhibitory on afferents, are under the influence of converging canal and/or vestibular activity (Blanks & Precht, 1976).

The movement plane correspondence between vestibular and neck afferents is striking. The identity of neck receptors involved is not clear. Joint receptors alone are probably not responsible, since neck muscle receptors have

been shown to converge with vestibular input in the posterior cerebellum (Schwarz et al., in press). It is quite possible that even other receptor types contribute. Although all possible care was taken to restrict the stimulation to neck afferents, our paradigm does not exclude excitation of receptors located elsewhere. However, if other receptors played a major role, the observed matching of plane specificity with horizontal canal input would only underscore the main conclusion of this study: one important aspect of sensory representation in the cerebellar cortex is movement direction and plane.

This aspect of the sensory projection seems, however, more readily available at the cortical input station than at the (more frequently studied) Purkinje cell output channels. Most of the described unitary patterns were found in the granule cell layer and are therefore probably unitary spikes from either granule cells or mossy fibers. This does not reflect the distribution of extracellular unitary spikes recorded. In fact most spikes were recorded in the Purkinje cell layer where they are much easier to isolate, these did, however, not yield the desired responses. The reason for this preference for granule cell layer response might be the anaesthesia or the immobilisation of the animal.

It may seem surprising that the vestibulo-cerebellum, so labelled by virtue of its direct connection to the labyrinth (Brodal & Hovik, 1964; Carpenter et al., 1972; Schwarz & Schwarz, in preparation), is not more involved in head rotation than other regions. Portions of the uvula on the other hand, not receiving direct vestibular fibers, appear to receive more head rotation information than other cortical portions. These aspects of our study should be compared with Haines' (1975) description of a rather extensive connection between the posterior cerebellar vermis and the vestibular nuclei in primates.

An interesting question is, whether the direction of a certain movement is represented in a defined topographical area of the cere-

bellar cortex. The great expanse of cortex receiving direction and plane specific information about horizontal head movements tends to argue against this assumption. It should however, be realized that head movements in pigeons represent a greater range of motor behaviour than in man: in addition to head rotations performed in a context analogous to human motor behaviour, the pigeon's head is rotated as response of the major vestibulo-visual reflexes, visual tracking reflexes, grasping movements and possibly other patterns. Thus different cerebellar regions, each responsible for the control of a separate pattern, might receive a distinct plane and direction specific input about head rotation.

ZUSAMMENFASSUNG

Neurone im posterioren Kleinhirn einschließlich des Vestibulocerebellum, die auf Rotation in der Ebene des horizontalen Bogenganges antworten, reagieren spezifischer für die Kanalebene als dies für primäre Afferenzen bekannt ist. Dies zeigt sich in einer Verminderung der Antwortamplitude, wenn die Drehebene aus der des Kanals geneigt wird, diese Verminderung ist ausgeprägter, als es der bekannten Cosinusfunktion entsprechen würde. Von 96 solchen Neuronen antworteten 1/3 auch auf Lateralebewegungen um Halsgelenke, nur drei Neurone reagierten auch auf Hals-Rollbewegungen. Neurone mit vestibulo-zervikaler Konvergenz fanden sich in Lobulus VI bis X, wobei sich die meisten dieser Zellen in der Uvula und sehr wenige im Nodulus fanden.

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VIRAL CULTURE AND ELECTRON MICROSCOPY OF GANGLION CELLS IN MENIERE'S DISEASE AND BELL'S PALSY

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Abstract. Specimens of Scarpa's ganglion during vestibular neurectomy were obtained in 6 cases of Meniere's disease and specimens of geniculate ganglion in 2 cases. Facial nerve decompression and studied by tissue culture methods for detection of possible herpes and cytomegalovirus infection. In electron microscopy inclusions in the form of interwoven yarn-like structures, coarse aggregates of chromatin and light nuclear bodies were found in several vestibular ganglion cells. No typical herpes virus inclusions could be demonstrated. The cultures of viruses all proved finally negative. At present there is no proof that viruses are present in Scarpa's or geniculate ganglions but the possibility remains that the inclusions observed might be viruses inactivated in the ganglion cells.

It has long been known that activation of herpes simplex virus frequently occurs following cranial root section (Carton & Kilbourne, 1952). Ellison et al (1959) found evidence of current herpes infection in 18 in a series of such patients and virus was isolated from peripheral lesion but not from two samples of geniculate ganglion. All patients possessed antibodies against herpes virus but there was no difference in antibody levels in those patients who developed herpes and those who did not.

It is generally accepted that virus travels retrogradely along the Schwann cells in the peripheral nerves, usually invading the geniculate ganglionic cell nuclei where the non-infective core of the virus may remain in a latent stage. On activation, new infective particles may be synthesized, released into cytoplasm and carried by means of the axonal flow to the epithelial surface where the vesicles are then produced (Paine, 1964).

Recovery of the herpes virus from the geniculate ganglion has now been successfully achieved. First Bastian et al (1972) isolated

herpes virus from 2 out of 23 trigeminal ganglia obtained at autopsy, viral cytopathic effects being evident after 3 weeks. Baringer & Swoveland (1973) were more successful isolating herpes virus from 6 out of 7 ganglions removed less than 12 h after death and maintained in culture for 10 to 45 days. Baringer (1974) also succeeded in culturing the virus from sacral ganglions innervating genitalia.

The concept of herpes virus infection of neural tissue has been extended to Bell's palsy, particularly as in 25% of the cases there are signs of cranial nerve polyneuritis in the form of transitory hypesthesia of the trigeminal nerve and in 43% involvement of the vestibular system (Adour et al, 1975). In 1972 McCormick put forward a hypothesis that a large percentage of cases of Bell's palsy could be caused by herpes virus taking up residence in the peripheral nerve cell axon where it was protected from neutralizing antibody and sensitized mononuclear immunocytes. Infectious virus particles might be present only during the first few days of paralysis and specimens for culture should be obtained early.

In a recent study on vestibular ganglion (Ylikoski et al 1977) structures resembling inclusion bodies were found in the nuclei of several ganglion cells in two patients with Meniere's disease. This prompted us to try to isolate viruses from vestibular and facial nerve ganglia which became surgically accessible at vestibular neurectomy or facial nerve decompression.

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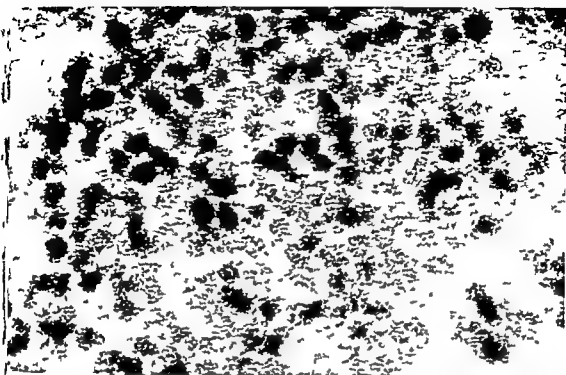


Fig. 2. Higher magnification of the yarn-like structure in Fig. 1. The thickness of the yarn is 20–25 nm and the yarn

appears homogeneous without peripheral rims and lighter centre. $\times 10000$.

Abbott Immunoglobulin (sheep) (Wellcome Reagents Ltd, Beckenham, Kent, England).

For electron microscopy the nerve specimens were immediately fixed in 0.1 M phosphate buffered 3% glutaraldehyde solution at 4°C for 2–8 hours, stored in sucrose post fixed in 1% osmium tetroxide and embedded in Epon. One micron thick sections were stained with methylene or toluidine blue *O* and studied under the light microscope. Sections for electron microscopy were stained with uranyl acetate and lead citrate and studied with JEM 100S or Hitachi HS 7S electron microscopes.

Paired samples from each patient were obtained and complement fixation antibody titres determined for viruses in the herpes group (influenza, para influenza, parotitis, morbilli, neo- and coxsackie viruses). Also titres for ornithosis, mycoplasma pneumoniae and toxoplasmosis were mostly included. None of the patients had any acute viral disease around the time of the surgery.

RESULTS

Viral cultures

In 2 patients with Meniere's disease a cytopathogenic effect was evident after 1 month of culture in HES cells. The morphologically changed cells formed foci in the cultures and the CPE resembled that of cytomegalovirus. Electron microscopy displayed no virus. The FA test for cytomegalo- and herpes viruses proved negative. The supernatants of the positive cultures were further transferred onto new HES cultures yielding no CPE, however.

It was suggested that the apparent CPE in the HES cultures was due to proliferation of ganglion cells from the biopsies.

Electron microscopy

Three kinds of inclusions were found in the nuclei of the vestibular ganglion cells.

1. Structures resembling interwoven yarn (Figs 1 and 2) in small round aggregates. Each



Fig 3 On the left the peripheral parts of the nucleus in a vestibular ganglion cell show coarse aggregates of chromatin among lighter chromatin material. The appearance of the complex might indicate degradation or

formation of the interwoven yarn like structures in Fig 2. On the right the cytoplasm is without any morphological changes. N=nucleus M=mitochondria x4000

aggregate was composed of 20–25 nm thick 'yarn' (3 dimensional impression) which in many places appeared contiguous and suggested that the aggregates could be composed of a single long structure. Cross sections of the yarn were seen in places. These were of homogeneous appearance and did not show a darker periphery (Fig 2).

2 Coarse aggregates of chromatin (Figs 3 and 4). At the periphery of the nuclei 20–60 nm particles of dark chromatin material could be seen. Between the coarse particles there was lighter chromatin material. Occasionally these coarse particles were seen at the nucleolus (Fig 4).

3 Light nuclear bodies (Fig 5). At low magnification these were seen as light areas in the granular chromatin of the nucleus. Higher magnification displayed hazy fibrillar structures arranged around a few interchromatin

granules. The diameter of these bodies was 0.8–1.0 μm (800–1000 nm).

DISCUSSION

The interwoven yarn like structures seen in the nuclei of vestibular ganglion cells share certain morphological features with microtubular inclusions, i.e. aggregates of tubular structures with a diameter of 22 nm. These inclusions are seen inside the endoplasmic reticulum (Uzman et al, 1971; Schaff et al, 1973; Bariety et al, 1974) and have a more distinctly tubular appearance than the structures we found in the nuclei. Originally it was believed that the microtubular inclusions were aggregated myxoviruses or paramyxoviruses. Later studies, however, have not been able to prove their specificity (Uzman et al, 1971; Bariety et al, 1974) or show that they con-

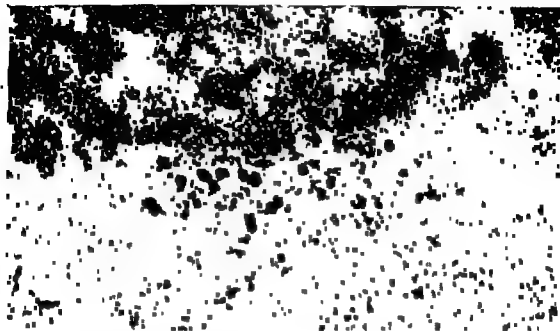


Fig 4 Coarse aggregates of chromatin at the nucleolus (above). Note the homogeneous appearance of the particles. They do not show peripheral membrane. $\times 80\,000$

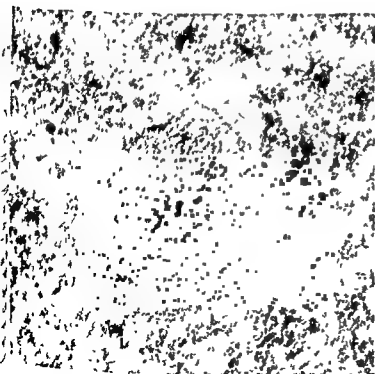


Fig 5 Light nuclear body in the nucleus. Note the delicately fibrillar periphery and the central interchromatin granules. $\times 80\,000$

nucleic acid (Schaff et al, 1973). In SSPE (subacute sclerosing panencephalitis), nucleocapsids which have a diameter of about 20 nm can occur in the nucleus (Raine et al, 1973, Iwasaki & Koprowski, 1974). However, also these have a more distinctly tubular appearance than the structures we found in the nucleus. So far there are no reports on studies that have been able to link corresponding structures with active herpes or cytomegalovirus infection. Of course, this also applies to the coarse aggregates of chromatin which do not show a constant diameter, suggesting that they are not viruses or parts of viruses.

Light nuclear bodies were first described by de The et al (1960) and Hinglais Guillaud et al (1961). These structures have attracted the attention of several investigators (e.g. Weber & Frommes, 1963, Bouteille et al, 1967, Krishan et al, 1967) and they are found in numerous tumours, virus infected cells and also under normal conditions. In no way do they appear to be specific to any virus. Nuclear bodies have been reported in studies involving viral activity (Dubois Dalcq et al, 1974) and Maiztegui et al (1975) showed that Argentine hemorrhagic fever, fluorescein labelled ascitic fluid, hyperimmune to Junin virus, was bound to these nuclear inclusions. One study of multiple sclerosis reported similar structures, which also contained nucleocapsids (Iwasaki et al, 1973). There is much to be said for the idea that the light nuclear bodies are structures linked with normal nuclear activity and one of the authors has found them in kidney biopsies in numerous kidney diseases (e.g. Runeberg et al, 1975).

However, it is possible that these structures could be activated in viral infections and under those circumstances they might produce viral components.

Our study could not link the nuclear structures with herpes or cytomegaloviruses. They might be linked with other viruses, even though earlier reports suggest that one of the inclusions can be found under normal conditions. It should also be borne in mind that

all the possible interrelationships between viruses and nerve cells are not known. Thus why we do not want to exclude the possibility that the structures observed could be connected by or could be linked with viruses inside ganglion cells (Waterson & Almon, 1969).

ZUSAMMENFASSUNG

Speziemen aus Ganglion Scarpa und Ganglion nodosum wurden in 6 Fällen von Menierescher Krankheit in Fällen von Facialisparalyse während der ersten Neurotomie bzw. totale Facialisdekompression genommen und mit Gewebekulturmethode für Herpes Zytomegaloviren untersucht. In elektronenmikroskopischen Untersuchungen der vestibulären Ganglien wurden drei Typen von Inklusionen gewundene fadenförmige Strukturen grobe Chromatinaggregate und kleine nukleäre Körper gefunden. Keine typischen Herpes oder Zytomegalovirus Viren wurden beobachtet. Es gibt keinen Beweis über das Faktum daß es keine Viren in Ganglion Scarpa oder Ganglion nodosum gibt, aber es ist möglich daß die Inklusionskörper inaktive Viren innerhalb der Ganglionzellen repräsentieren.

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SIALOCHEMICAL EXAMINATIONS IN NON-TUMOROUS PAROTID ENLARGEMENTS

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Abstract Secretion rates and the composition of isolated

of the results indicated that sialochemical examination may provide a valuable help in the differential diagnosis of parotid diseases characterized by swelling of the gland. Chronic parotitis is characterized by a significantly decreased flow rate, extremely high sodium and protein concentration and lack of secretory response to stimulation. In Sjögren's syndrome the low flow rate was found to be associated with a decreased potassium secretion rate as well as decreased flow rate and decreased sodium concentration after stimulation. In sialosis where flow rates display a considerable individual variation a significant increase in potassium secretion rate could be revealed.

Enlargement and hypofunction is a common symptom of many diseases of the parotid gland. Tumours may often be excluded by means of clinical history, palpation and sialography. On the other hand, correct diagnosis may be hindered by non-tumorous swellings of the salivary glands.

The chronic recurrent parotid swellings most frequently encountered in clinical practice may be classified into three groups, namely chronic recurrent parotitis, rheumatoid sialadenitis (Sjögren's syndrome) and sialosis. Although distinction of these enlargements is important both for therapy and prognosis, clinical history and examination are often insufficient to make this distinction. Furthermore, sialographic examination often fails to establish a diagnosis in many cases, since radiographic alterations are frequently missing or are not specific for the particular

pathological states (Diamant & Fendler, 1959; Rauch & Gorlin, 1970; Chisholm et al., 1971; Chisholm & Mason, 1973).

There have been attempts for a long time to draw diagnostic conclusions from the changes in exocrine function of the gland. The sialometric and sialochemical changes observed in various non-tumorous parotid diseases have been surveyed by Rauch (Rauch, 1949; Rauch & Gorlin, 1970). Although some parotid diseases are associated with characteristic changes in secretion rate or in the salivary concentration of some ions and protein, estimation of salivary gland function does not belong to the diagnostic means. This may be accounted for both by the tedious character of these examinations and the frequent contradictions of literary data. These conflicting observations may be explained by differences in sialochemical collection methods as well as the non-standardized physiological and environmental factors affecting the function of salivary glands.

In our previous reports (Benedek-Spät et al., 1976a, b) an account has been given on parotid flow rate and parotid salivary composition in the resting state and after stimulation as observed in healthy university students. Our studies on parotid function in Sjögren's syndrome have revealed that, besides a decreased secretion rate and increased protein concentration of saliva, the syndrome is characterized by a diminished responsiveness to stimulation (Benedek-Spät et al., 1979).

The present work was designed to reveal changes in flow rate or composition of par-

■ 1 Distribution of the examined subjects according to diagnosis, sex and age

	Number of patients	Sex		Age (years)	Histological verification
		Male	Female		
all	17	5	12	51±3	—
chronic parotitis	6	2	4	52±4	—
Sjögren's syndrome	21	4	17	49±3	8
salivary gland disease	24	11	14	50±2	3

in three forms of parotid swelling, chronic recurrent parotitis, rheumatoid sialoadenitis and sialosis.

METHODS

The examinations reported here have been carried out in 68 subjects. Diagnosis, sex and age of the patients are shown in Table I. For control purposes we examined subjects who did not suffer from any systemic or salivary gland disease and did not take drugs.

Diagnosis of chronic recurrent parotitis has been based on clinical history as well as on histological and sialographical examinations.

Diagnosis of Sjögren's syndrome has been established on the characteristic clinical signs and laboratory data and, in about one third of the cases on histological examination. Some patients with verified Sjögren's syndrome had swollen parotids at the time of sialochemical examination. Considering, however, that there is a difference in respect of parotid function between these patients and those having parotid swelling, all these data were pooled into one group.

Table II shows the diseases associated with sialosis, usually preceding the bilateral parotid swelling in our sialotic patients. Sialography failed to reveal any significant alteration in these patients. Histological examination verified the diagnosis in three cases.

Saliva was collected in the fasting state, between 8.00 and 9.00 a.m. by means of a thin polyethylene tube introduced into one or both parotid ducts. Details of the technique have been previously described (Benedek Spät,

1973 a). Unstimulated saliva was collected for 10 or 20 min, depending on the flow rate. After an interval of several minutes the secretion was stimulated by a 2% solution of citric acid, of which two drops were applied every half minute on the upper surface of the tongue. Stimulated saliva was collected for 3 to 5 minutes. No chemical analysis could be undertaken when the volume of saliva was below 50 µl.

Secretion rate, both before and after stimulation, was measured and the samples were analysed for sodium, potassium and protein concentration by methods previously detailed (Benedek Spät, 1973 a). Amylase activity was estimated with Phadebas Amylase Test (Pharmacia).

Mean values and standard error of the mean (S.E.) were calculated. With regard to the fact that only unilateral examinations were undertaken in some patients, while bilateral examinations were executed in others, standard methods used for statistical calculations have been correspondingly modified.

Table II Associated diseases of 24 sialotic patients

Diabetes mellitus	9
Hypothyroidism	3
Oligomenorrhea or dysmenorrhea	5
Long term steroid treatment	2
Chronic alcoholism	2
Chronic hepatitis	1
Iron deficiency	1
Bronchial asthma	2
High blood pressure	6
Neurotic complaints	9
Rheumatoid arthritis	2

diseases examined by us Cannulation of the gland helped the diagnostic work also in cases where measurable amounts of saliva could not be collected, as it provided a sample for bacteriological examination

Swollen parotids, generally on both sides, may often be encountered in Sjogren's syndrome, a kind of autoimmune disease Such a swelling presents no diagnostic problem when the characteristic clinical symptoms and laboratory changes are manifest (Bunim et al , 1964, Rauch, 1966, Whaley et al , 1969, Cummings et al , 1971) Not infrequently, however, a patient will complain of a swollen gland or hyposalivation when the characteristic clinical symptoms have not yet developed, and pathological changes cannot yet be detected in the sialogram (Chisholm et al , 1971, Chisholm & Mason, 1973, Encson, 1974) On the basis of a few reports dealing with the sialochemical changes in Sjogren's syndrome it is accepted that, in common with other infectious inflammations, hyposalivation also in this disease is accompanied by a high sodium concentration (Rauch, 1959, Mandel & Urmash, 1973, 1976) Our earlier study on this disease (Benedek-Spat et al , 1975) indicated that the secretion rate might depend on the progression of the disease The sodium concentration of the saliva depended on the flow rate we observed high sodium values in cases only where the flow rate was below 0.01 ml/min Normal sodium concentration values indicate that the sodium reabsorbing capacity of the duct epithelial cells is not severely impaired in rheumatic inflammation The extremely high sodium concentration values found in cases of very low flow rate may be accounted for by sodium re-equilibration in the more distal region of the excretory duct (cf. Schneyer et al 1972 Young 1973) The present examinations confirm these findings on the basis of a larger number of observations Despite some high sodium values found in cases of extreme hyposalivation the mean sodium concentration did not differ significantly from the control Characteristically for

Sjogren's syndrome, we found an increase in concentration of protein and increased amylase activity, besides the well-known decrease in secretion rate For distinction between Sjogren's syndrome and sialosis the protein secretion rate below 1 μ Eq/min in the E group may be of great importance (Fig. 2)

Sialosis is the term used to describe a non-inflammatory, non-neoplastic reversible bilateral swelling of the salivary glands In majority of reported cases have been related to hormonal or neural disorders, malnutrition, cirrhosis of the liver, and chronic alcoholism (Rauch, 1959, Seifert, 1971, Seifert & Dees 1975, Donath, 1976)

The mean secretion rates in our sialotic patients were significantly higher than in controls This increase in secretion rate is, however, of no diagnostic significance as the range of individual observations extends from very low to very high values (Fig. 3) Sialosis is generally thought to be characterized by low flow rates (Rauch 1966) Nonetheless, high values for secretion rate were reported in sialotic cases of neurogenic origin (Rauch, 1959) or alcoholic origin (Abelov et al , 1976) as well as in the early stage of disease (Diamant, 1974) In agreement with these observations, 5 of our 8 highest rates were obtained from chronic alcoholic or neurotic patients Nevertheless even despite these high values we do not regard a decreased flow rate as characteristic for sialosis Conflicting opinions in this respect may be attributed to the dependence of flow rate on the progression of the disease or the administration of different drugs because of an associated disease

An increased potassium concentration in the parotid saliva of sialotic patients was reported by Rauch (Rauch 1956 1966) but we were unable to observe such an increase The discrepancy between these results may perhaps be attributed to the same factors discussed for the flow rate More important, however, is the observation that the potassium secretion rate is much higher than

Sjögren's syndrome (Fig 2). Consequently this parameter would seem to be a useful basis for differential diagnosis. Stimulation with citric acid is followed by a characteristic increase in secretion rate and in sodium concentration of the saliva in healthy subjects. Stimulation failed to augment the secretion rate in chronic recurrent parotitis. It was painful in some patients. While sialotic patients responded to stimulation in a manner comparable to healthy controls, the stimulated flow rate in Sjögren's syndrome was significantly smaller than in controls, as well as in sialotic patients. The observation of a low flow rate in Sjögren's syndrome is in agreement with the reports of Seward et al (1966) and Chisholm & Mason (1973). While a low flow rate does not mean a decreased responsiveness to stimulation (the ratio of stimulated to unstimulated flow rate is comparable to that in healthy subjects) there is a reduction or even a lack of response in respect of sodium concentration. Since the sodium response is maintained in sialosis, examination of sodium concentration before and after stimulation may, again, be of diagnostic importance.

ACKNOWLEDGEMENT

For statistical advice of Dr J. Fischer, Institute for Computing Techniques and Automatization, Hungarian Academy of Sciences, is gratefully acknowledged.

ZUSAMMENFASSUNG

Es wurde bei 51 an chronischer rezidivierender Parotitis, Sialose oder Sjögren-Syndrom leidenden Patienten und bei 17 gesunden Kontrollpersonen das Maß der isoherten Speichelsekretion und die Zusammensetzung der Speichelsekretion untersucht. Die Bewertung der Ergebnisse ergab, daß die sialochemische Untersuchung in der Klärung der Speicheldrüsengeschwulste verschiedenen wertvollen Hilfen leisten kann. Die chronische Parotitis ist von der signifikant verminderten Sekretion und der äußerst hohen Natrium- und Proteinkonzentration ferner vom Mangel der auf Stimulierung gegebenen sekretorischen Antwort charakterisiert. Im Sjögren-Syndrom war die niedrigere Speichelsekretion von verminderten Kalium-Sekretionswerten begleitet und nach Stimulation war das Maß der Sekretion und die Natriumkonzentration signifikant kleiner als bei den Kontrollwerten.

Im Sialose war außer der individuellen Schwankung der Sekretion die signifikante Zunahme der Kaliumsekretion charakteristisch.

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THE MUCOCILIARY ACTIVITY OF THE UPPER RESPIRATORY TRACT

II A Method for In Vivo Studies on Maxillary Sinus Mucosa of Animals and Human Beings

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Abstract A method for photoelectric in vivo recordings of the mucociliary activity of the mucosa of the upper respiratory tract is described and tested in model experiments. In vivo recordings from the maxillary sinus mucosa of rabbits were easy to analyse and the method is useful for further experimental research on animals. Examples of in vivo recordings from the human maxillary sinus during Caldwell-Luc operations are also presented. These results are compared with subsequent in vitro recordings of biopsy material.

Several authors have made human in vivo studies of mucociliary transportation by depositing tracer substances in the nose (Hilding, 1932, Ewert, 1965, Proctor & Andersen, 1976), maxillary sinuses (Yates, 1924, Hilding, 1944, Jøsserkløpper, 1966) and the middle ear (Sade, 1967).

In pathological conditions retardation and stagnation of secretions have been observed. Whether this is due to changes in the amount and tenacity of secretions or impaired function of cilia is not easy to evaluate although vigorous activity of cilia has been observed in sinusitis even in the presence of 'overwhelming infection of long standing' (Proetz, 1933).

The pattern of cilia movements within the mucous layer can be assessed from periodic changes in light reflections observed on the surface of the mucous membrane through an operating microscope (Frenckner & Richtner, 1939). Recording methods allowing direct evaluation of the frequency of this surface activity have been improved during the last decade (e.g. by Håkansson & Toremalm (1965), Guil-

lem et al (1965), Chevance & Lennon (1970) and Mercke et al (1974). Use of these methods has hitherto been limited to experimental in vitro studies.

Simplified equipment for recording local mucociliary activity has been developed and technical data as well as preliminary in vitro recordings from human material have already been described (Reimer et al 1977).

The aim of this paper is to report on the possibilities and limitations of the method in use on the maxillary sinus mucosa of rabbits and human beings in vivo.

MATERIAL AND METHODS

The mucociliary activity is recorded indirectly via the variations of surface light reflections brought about by the movements of cilia. The reflected light is focused by an operating microscope (Zeiss OPMII), transformed to electrical signals by a phototransistor (Type FPF 139) and recorded by an ink writer (Elema Schonander Mingograph 34).

An oscilloscope facilitates focusing on a reflecting area of the mucosa. A bandpass filter (3-30 Hz) suppresses high frequency interference. The recordings represent the light reflections from a circular mucosal area about 0.1 mm in diameter. The method and equip-

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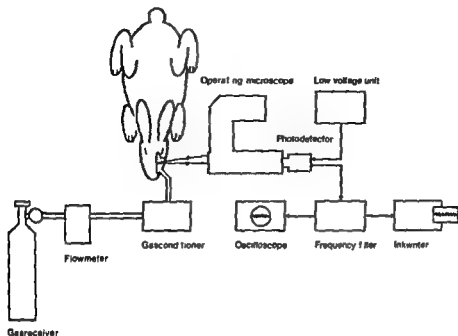


Fig 1 C
for in vivo experiments
rabbit maxillary sinus

ment has been described in detail in a previous paper (Reimer et al, 1977)

Model experiments

The frequency and amplifying characteristics of the equipment were tested experimentally by recording from the illuminated surface of a drop of oil spread over a piece of plastic placed on a vibrating loudspeaker membrane. In a series of experiments tracheal specimens from rabbits with active ciliary movements were recorded from when absolutely still, and when moved by the loudspeaker membrane vibrating at different frequencies and amplitudes.

By altering the temperature of the specimen

different mucociliary wave frequencies were achieved. The induced vibrations were adjusted to a recorded amplitude of 1-2 times that of the mucociliary recording.

Animal recordings in vivo

Rabbits (weight 2-4 kg) were anaesthetized with Nembutal® intravenously (40 mg/kg initially, thereafter approximately 20 mg/kg). A hole of about 5×5 mm was made in the alveolar ridge and the maxillary sinus was exposed. The medial wall was illuminated and a reflecting surface of about 2-4 mm² was focused. The mechanical stability of the rabbit head was assured by a special holder. The head could be moved in any direction by means of micrometer screws. Conditioned air was introduced to the sinus via an additional hole. The experimental set up is shown in Fig 1 C and examples of recordings in Fig 3. On account of accumulations of mucus or to unavoidable movements of the equipment during the experiments, minor adjustments of focus were sometimes necessary. The range of frequencies was studied on three reflecting areas exposing a greater part of the medial wall of the sinus.

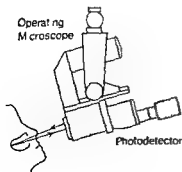


Fig 2 Operation situation for clinical in vivo recordings

recording in vivo

patients were under general anaesthesia (halothane®) and intubated orotracheally. The patient rested in a circular sandbag on an ordinary operating table. The clinical application is illustrated in Fig. 2. Since the original method was designed for an exact replica of an adult maxillary sinus (Reimer et al., 1977), the microscope and the ancillary equipment could be readily adapted for *in vivo* studies in the operating theatre. The microscope was enclosed in a sterile bag. The phototransistor is small and light that it can easily replace one of the microscope eyepieces without disturbing vision through the other. After careful anaesthesia the antrum was opened *ad modum* (Jedwell & Luc, 1977). With a magnification of about 100×, a light reflecting area on the posterior wall could be kept in focus and the mucociliary activity recorded. Accumulation of mucus or blood had to be cautiously sucked away occasionally. After the *in vivo* recordings the tracheal part of sinus mucosa was carefully explanted and within about 20 min placed in an experimental chamber with the size and configuration of an adult maxillary sinus. Record-

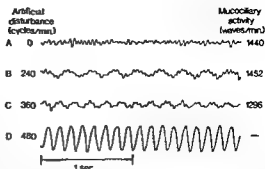


Fig. 4. Recordings from a tracheal specimen placed on a vibrating loudspeaker membrane. The added movements are easily recognized (B and C). When the amplitude of the induced vibrations is too large the mucociliary waves are concealed (D).

ings were commenced 10–15 min later at an ambient temperature of 37°C and relative air humidity of >80%.

Control recordings

Possible sources of disturbance are movements by the patient (muscular, cardiovascular and respiratory trembling) and by the operating microscope. The mechanical disturbances *in vivo* were checked by recording the light reflected from a piece of aluminium foil placed on the forehead of the patient. The mechanical stability *in vitro* was tested in the same manner from a piece of aluminium foil placed in the bottom of the experimental chamber.

RESULTS

Model experiments

A white piece of plastic, covered with a drop of only liquid, offers a smooth surface without irregularities. The frequency of the recording follows the frequency of the induced vibrations exactly (Fig. 3 upper). The attenuated amplitude at frequencies above 30 Hz is due to the filter. The effect of the filter on recordings from a surface vibrated with constant amplitude at different frequencies is shown in Fig. 3 lower.

Recordings from a mucous membrane with active cilia are seen in Fig. 4. When the mu-

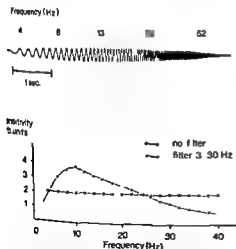


Fig. 3. Recording from a light reflecting surface on a loudspeaker membrane vibrating with constant amplitude from 1 to 80 Hz (upper). The amplitude at frequencies above 30 Hz is attenuated by a filter. Relative sensitivity at different frequencies tested in the same manner with and without the filter (lower).

Table I Frequencies (waves/min) based on recordings of 10 sec duration

Expt no	Time (minutes)				
	0	1	2	3	4
<i>No artificial disturbance</i>					
1	1 398	1 380	1 344	1 350	1 350
2	1 410	1 404	1 416	1 422	1 422
<i>Artificial disturbance (cycles/min)</i>					
	0	240	360	480	0
3	720	666	720	882	720
4	858	954	954	846	801
5	990	1 032	948	870	1 008
6	1 284	1 266	1 254	1 170	1 296
7	1 446	1 404	1 350	1 320	1 380

cous membrane is vibrated at 240 to 360 Hz, an extra undulation is seen in the recording. The frequency of the mucociliary waves can still be calculated but an error is introduced (Fig 4C). If the amplitude of the extraneous movement is too great (Fig 4D), the mucociliary waves will ultimately be swamped and the recordings no longer represent the movements of cilia. The results of the recordings at different mucociliary wave frequencies and a disturbance amplitude of 1–2 times that of

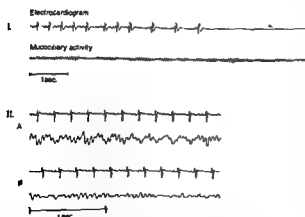
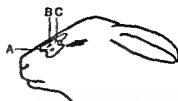


Fig 5 Recordings from the maxillary sinus of rabbits I A regular wave movement and amplitude modulation during the final heart beats after an overdose of Nembutal® II Recording of mucociliary wave movements and amplitude modulation



Area	Point	Frequency (waves/min)
A	1	1416
	2	1422
	3	1452
B	1	1437
	2	1374
	3	1434
C	1	1182
	2	1188
	3	1296
	4	1308

Fig 6 The range of frequencies on three light areas on the medial wall of a rabbit maxillary sinus is outlined in natural size related to the head

the mucociliary wave recording is given in Table I. The error introduced by such disturbance gives no remarkable deviation from the expected frequency.

Animal recordings *in vivo*

In Fig 5, I, a recording during the final heart beats from an animal killed with an overdose of Nembutal® is shown. There was no immediate change in character or frequency of the mucociliary light reflections. In some bits a low frequency undulation of the recordings was seen (Fig 5, II A) which disappeared after a minor adjustment of the rabbit (Fig 5, II B). The simultaneous electrocardiogram shows that the cardiac rhythm is not disturbed by this disturbance, which is thus probably derived from pulsations of the vascular bed. This disturbance is easily recognized and does not interfere with the calculation of the mucociliary activity.

The range of mucociliary wave frequencies from different reflecting parts in one rabbit is shown in Fig 6. It is seen that the range of frequencies from one reflecting area is small, but great differences can be seen between different

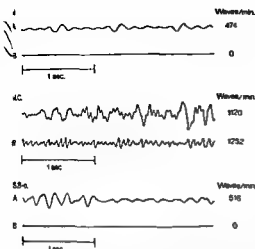


Fig. 7. Control recordings from a piece of aluminium foil on the forehead of a patient during apnoea (A) and in the mucous membrane in vivo (B) in a case of purulent sinusitis (text). S B O. Recordings from the mucous membrane in vivo (A) and in vitro (B) in a case with apparently no mucociliary activity (see text).

Technical recordings in vivo and in vitro

Acceptable conditions regarding extraneous disturbances were achieved only during apnoea. Extraneous disturbances are recorded from the aluminium foil on the forehead of a patient (Fig. 7, Control, A). A corresponding in vitro recording shows complete absence of disturbances (Fig. 7, Control, B).

Two combined in vivo and in vitro recordings are shown below. The first patient, M C, had a long standing purulent sinusitis. In vivo recordings from the posterior wall show a nodic movement, which is disturbed by irregular waves of larger amplitude. It was possible to count the periodic movements during 1 sec of the recording. The calculated frequency was 1120 waves/min (Fig. 7, M C, A). In vitro recording from the same part of the mucosa revealed a regular wave movement of 1252 waves/min (Fig. 7, M C, B). In the next case (S B O) a cyst occupied the antrum. There was no visible mucociliary activity on the posterior wall. The recording from the illuminated mucous membrane (Fig. 7, S B O, A) probably only represents movements

of the patient and/or the operating microscope since the corresponding in vitro examination confirmed the absence of mucociliary activity (Fig. 7, S B O, B).

DISCUSSION

The importance of experimental and clinical research regarding the local mucociliary activity of the extrathoracic airways has been discussed in previous papers (Mercke et al, 1974; Reimer et al, 1977).

The mucous membrane in these regions constitutes an ideal object for functional studies, since the cilia continue to work for several hours even after removal from the body. This activity can therefore be studied in vitro under carefully standardized conditions. However, in vivo studies are also needed, for example to find out how far the mucociliary activity is depended on a continuous vascular supply and neural control.

The light intensity recorded from an illuminated part of the respiratory mucous membrane can fluctuate due to movements from different origins (Toremalm et al, 1974). During in vivo conditions, cardiovascular and respiratory trembling may disturb the recording of the movements produced by cilia and mucus. The influence of extraneous movements has therefore been analysed experimentally.

The model experiments showed that mucociliary wave movements can be recorded in directly even from an object which is not absolutely still, provided the extraneous disturbances are within certain limits related to the mucociliary recordings. During animal experiments, mucociliary activity recordings sometimes exhibited irregular low frequency events which coincided with simultaneously recorded cardiac beats. This activity varies from place to place in the same sinus, and is probably derived from pulsations in the vicinity of the vessels (Fig. 5, II). The recordings from specimens during induced vibration support this hypothesis (Fig. 4, B and C).

In vivo recordings from the rabbit maxillary sinus were easy to analyse, and the frequency range of each examined area was small (Fig. 6). These facts make this application of the recording method very useful in experimental research.

The recordings from humans had to be obtained during fairly short apnea periods of 20–30 sec, since the head of the patient could not be fixed as effectively as the head of an animal. The mechanical stability of the operating microscope fixed on a floorstand is quite good but cannot be compared to the rigid laboratory equipment. This is seen in the result of the control recording from a piece of aluminium foil on the forehead of a patient (Fig. 7, control, A). In addition, inflammation of the mucous membrane presumably brings about more intense vascular pulsations than in healthy tissues. We attribute the irregular low-frequency background activity seen in the in vivo recording of case M. C. (Fig. 7, M. C., A) to these factors. The in vivo recording from other case (Fig. 7, S. B.-D., A) represents irrelevant extraneous movements only, the corresponding in vitro examination revealed no mucociliary activity. Inflammatory diseases of the paranasal sinuses and the middle ear are often characterized by retention of secretions. It is therefore of great interest to elucidate the functional state of the ciliated epithelium in such disorders.

Further combined in vivo and in vitro examinations of the human maxillary sinus, as well as extended functional and morphological studies on other human specimens from the upper respiratory tract, will be reported in a forthcoming paper.

ZUSAMMENFASSUNG

Beschreibung einer fotoelektrischen Methode zur Registrierung in vivo der mucociliaren Schleimhautaktivität.

Sonders für weitere Tierexperimentelle Forschungszwecke Als Beispiel werden auch in vivo Aufzeichnungen

von menschlichen Kieferhöhlen während des Apnoe nach Caldwell-Luc dargestellt. Diese Resultate werden mit anschliessenden in vitro Aufzeichnungen an humanem Material verglichen.

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EXPERIMENTAL SURGERY ON THE NOSE

I Airflow and Goblet-cell Density

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Abstract. In rabbits the nasal vestibule was surgically occluded on one side. The rabbits were observed for varying periods, maximum 90 days. The mucosa of the septum was removed from both sides, stained by the PAS alcin blue whole mount method, and the goblet cells were counted. The difference in goblet-cell density between the occluded and open side of the nose was less than expected according to previous reports. A completely occluded air flow for 3 months caused only some increase in density, doubling of the air flow some decrease in goblet-cell density. During the first 16 days there was an increase in density on the occluded as well as on the open side of the nose. This can hardly have been due to increased airflow, but must be attributed to an irritative action of the operation upon the nasal mucosa.

One of the most important functions of the nose is to protect the lower air passages. Mucus production and ciliary function are important factors in conditioning the air passing through the nose. Mucus forms in the goblet cells, intranasal mucosal glands, and in the submucosal, tubular, and alveolar glands. The activity in the submucosal glands is accelerated by parasympathetic stimulation (Ånggaard, 1976) and by oestrogenic substances (Helmu et al., 1975; Mortimer et al., 1936). However, studies on the functional role of goblet cells in mucus production are lacking, and we possess only limited knowledge of the factors which influence their number, activity, and turnover in the nose. Hilding and his associates (1932, 1970, 1970) carried out pioneer animal experiments for the purpose of elucidating the role of the air flow in the morphology of the nasal mucosa. By unilateral, surgical occlusion of the nasal vestibule they demonstrated in rabbits an increase in goblet cells on the occluded side and meta-

plasia of the respiratory epithelium towards stratified squamous epithelium on the open side. The interesting studies of Hilding et al. were based upon an estimate of the quantitative increase in goblet cell density, and they only state the time of this increase.

In clinical practice, we often come across differences in the passage of air through the two nostrils without being able to demonstrate any definite macroscopic differences in the mucosa. It has also been demonstrated (Proetz, 1953; Masing, 1967) that the mucosa on one side of the nose is not uniformly affected by the inspired air and that in certain parts of the nose the air flow is stronger than in others. Differences in the strength of the air flow constitute a physiological phenomenon. Therefore, the mucosal changes that they possibly cause may be imagined to be so subtle that they are difficult to record with reasonable accuracy by a subjective estimate of conventional histological sections. Objective, quantitative studies are needed. They can be performed on the epithelial goblet cells by whole-mount methods (Moe, 1955) in which the epithelium is stained by PAS (Moe, 1955), or by PAS alcin blue (Kessing, 1968; Tos, 1970), and the goblet cells are counted on the surface in several, well-defined fields.

If the pathophysiology of the nose is to be better understood, it is important to establish the extent to which the mucosa is altered by a reduced, abolished, or increased air flow, when this change occurs, and by what factors, other than the air flow, it is influenced.



Fig 1 Closed and well adapted right nasal vestibulum (a) Frontal (b) lateral view

MATERIAL

Rabbits of the type Copenhagen White from Statens Seruminstitut, Copenhagen, were used. The rabbits were adult, weighing about 3 kg. Body weight was recorded before the operation and at sacrifice, no rabbit had lost

The material was divided into three groups (Table I): 1 Normal rabbits (11) living under the same conditions as the others; 2 Anaesthetized rabbits (7) matched to each

group of operated rabbits in order to avoid the influence of anaesthesia; 3 Operated rabbits (20) whose right nostril was occluded so that respiration was exclusively through the left one.

METHOD

All operations were carried out under general anaesthesia using i.v. Nembutal. Rabbits with extraordinarily active movements of the

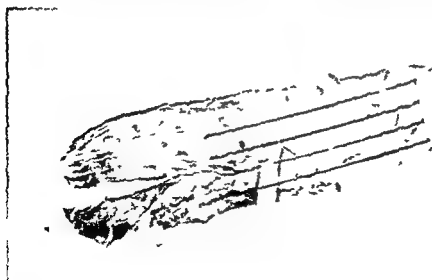


Fig 2 Whole mount preparation of right nasal septum. The part in A

horizontal biopsy has been taken from the anterior part and a vertical biopsy from the posterior part of the respiratory region *2

Table I Number of animals in each group Number of rabbits which for different reasons should be excluded from the operated material

Group of observation (s)	Normal animals	Anaesthetized animals	Operated animals	Discharged animals
2		1 1 1	3 3 3	1 catarrhalia 1 right side
		1	1	
3		1	2	1 defect closure
3		1	3	2 catarrhalia 1 left side 1 catarrhalia
3		1	3	
11		7	20	

s and with their paws they will try to remove any foreign body in this region (if both nostrils are occluded, they die of respiratory failure). After testing various surgical procedures, we selected the following. A couple of mm from the external orifice an oval incision is made in the entire circumference of the alar sulcus. The mucosa was undermined, incised, and the margins from the medial and lateral side sutured with submucous sutures of absorbable material. The skin was carefully closed with silk sutures (Fig. 1). Two factors seem to be of particular importance in securing stability of the operation. To avoid inflammatory reaction the sutures in the mucosa must be submucous and must not penetrate into the nasal cavity. It is very important

also to undermine the mucosa in a considerable area, as a taut mucosa over the medial, cartilaginous part of the septum will easily lead to perforation.

The rabbits were killed with an overdose of Nembutal i.v. After sacrifice, the nasal septum was removed *in toto* and fixed in formalin alcohol. The entire mucosa on both sides was removed from the underlying, cartilaginous part of the septum, microdissected with removal of the deeper part of the lamina propria, stained with PAS-alcian blue (Tos, 1970), and embedded in a chamber with a solution of anise oil-colophonium (Fig. 2).

Goblet cells were counted in a Reichardt projection microscope, magnification 500 \times , in fields measuring 0.01768 mm². Four equidis-

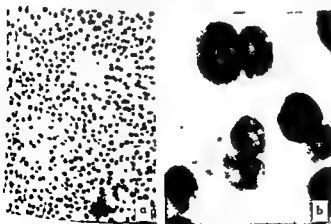


Fig. 3 Distribution of goblet cells seen in whole mounts PAS-alcian blue staining (a) $\times 80$ (b) $\times 800$.

Table II *Individual median density of goblet cells (cells/0.01768 mm²) in the right and left sides of the septum from normal, anaesthetized and operated rabbits*

Observation period (days)	Normal		Anaesthetized		Operated	
	Right	Left	Right	Left	Closed Right	Open Left
0	40 77	20 76				
4			46	44	47 59 95	47 47 55
6			25	66	54 59 113	47 80 88
10			84	46	104	45
16						81
20					110	83
			34	41	52	18
					76	42
30	32 55 69	50 63 76	25	86	46	43
60	35 65 71	38 61 79	78	65	84	
	11 55 58	23 44 86	76	55	59 64	20 51
	55	61	46	55	62	47
Median	57		50			

tant, horizontal lines were drawn on the slide, and every tenth field along these lines was counted, starting anteriorly at the junction to the vestibule (Fig. 2)

RESULTS

Four of the operated rabbits had to be excluded from the quantitative analysis because of catarrhalia and one because of a minor defect in the closure (Table I). Moreover, one right and one left septal mucosa had to be excluded for histotechnical reasons.

Morphology On the whole mounts the goblet cells stood out as bluish sharply defined, round to oval spots on a paler bluish-green background (Fig. 2). In normal rabbits the size varied only slightly around 8 μ m in

diameter, but scattered in the epithelium were also goblet cells measuring 10–12 μ m, even 20 μ m in diameter.

The distribution was irregular, manifested itself as differences in the density within the same counting field, between various neighbouring fields, and between different septa. This made for a marked dispersion of the density for each septum and emphasized the need to count in many fields to obtain a reliable estimate of the median density, and it also goes to show that an estimate of the density on the basis of histological sections can afford no definite expression of the density.

It was estimated that the ratio between small and large cells was the same on the closed and on the patent side, as in normal rabbits.

Table III Individual median density of goblet cells (cells/0.01768 mm²) in the 12 anterior and posterior fields of the septum from normal anaesthetized and operated rabbits

Right L=left Ant=anterior Post=posterior

Experiment (no.)	Normal				Anaesthetized				Operated			
	Ant		Post		Ant		Post		Closed (R)		Open (L)	
	R	L	R	L	R	L	R	L	Ant	Post	Ant	Post
1	66	23		14								
2	49	88	85	70								
3					45		31		93	83	21	50
4						57		34	27	52	47	38
5									46	71	43	56
6					27		37		69	67	63	80
7						86		66	125	101	73	81
8									84	69	16	53
9					39		100		■	■	109	72
10						43		75			47	56
11									172	94	74	64
12					35		43		65	67	50	38
13						43		45	102	53	12	53
14	94	53	60	63	20		46		91	31	58	39
15	14	27	65	48		124		68				
16	69	31	18	106								
17	83	90	61	72	100		86		94	65		
18	52	72	79	59		42		73				
19	32			45								
20	67	40	38	44	46		77		64	62	23	24
21	89	112	36	44		53		60	60	55	66	29
22	64		32	42								
23	64	53	60	48	39	53	46	66	86	67	49	53
24		56		54		44		63		77		51

Density of Goblet Cells

Normal material The individual median density of each septum, based upon counting about 30 fields, varied somewhat from nose to nose (Table II) from 11 to 86 cells/field. There was no significant difference in interindividual median density between the right and left side (Mann-Whitney, $p > 0.1$).

The interindividual median density of the anterior part of the septum, based in the case of each septum upon 12 anterior fields, was 66 cells/field as compared with 54 cells/field in 12 posterior fields (Table III). This difference was not statistically significant ($p > 0.1$).

Anaesthetized material There was no significant difference in the interindividual goblet-cell density between normal and anaesthe-

tized rabbits ($p = 0.8$) or between the right and left septum ($p > 0.7$) (Table III) or between the anterior and posterior segment of the septum ($p > 0.1$, Table III).

Operated material

1 Occluded versus normal material The interindividual density of all septa from the occluded side was significantly higher ($p < 0.03$) than that of all normal septa (right and left). The median density of the anterior segments of the septum was not significantly higher than of the posterior one ($p > 0.05$, Table III). The anterior and posterior segments of the septum respectively showed a significantly higher density ($p < 0.025$) than the corresponding segments in the normal material.

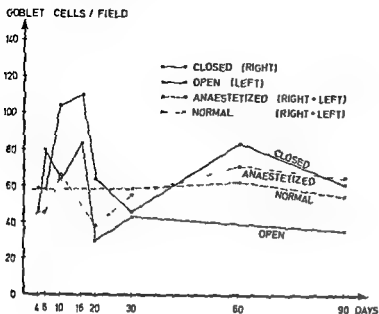


Fig. 4. Interindividual median density curves including all counting fields from each septum in different materials.

2 *Open side versus normal material* The interindividual density of all septa on the open side was not significantly lower ($p > 0.05$) than that of all septa (right and left) from the normal material (Table III). On the open side there were no differences in density between anterior and posterior segments of the septum ($p > 0.1$, Table III). The anterior and posterior segments of the septum respectively showed no significant difference in density as compared with the corresponding segments in the normal material ($p > 0.05$).

3 *Occluded versus open side* The interindividual density of all septa on the occluded, right side was significantly higher ($p < 0.01$) than of the open side. The density in the 12 anterior fields on the occluded side was significantly higher ($p < 0.01$) than in the corresponding anterior fields of the open side. Similarly, the density in the 12 posterior fields on the occluded side was significantly higher ($p < 0.025$) than in the corresponding fields on the open side.

Density in Relation to Observation Period

Within the normal and anaesthetized materials no essential changes in density occurred during the observation period (Fig. 4). Among the operated rabbits there occurred on the oc-

cluded side a distinct increase in density during the first 16 days, followed by a gradual decrease until the 20th day, by which the density had approached normal. Then there was a slight increase, so that after 90 days the density of the nostril was only a little higher than normal.

Surprisingly, the open side also showed a considerable increase in density up to the 16th day and thereafter a decrease until the 20th day. After 90 days, the density was only a little lower than normal (Fig. 4).

Therefore, the changes in goblet-cell density were assessed separately for the period 20-90 days.

The interindividual density during the period 20-90 days after the operation was 62 cells on the occluded side as compared with 60 cells/field in the normal material. This difference is not significant ($p > 0.1$). On the open side the density during this period was 42 cells/field, which is significantly lower ($p < 0.001$) than in the normal material. During the observation period, the density on the occluded side was significantly higher than on the open side ($p < 0.001$).

To ascertain whether the change in density occurs predominantly in the anterior or posterior segment, the density during the period 20-90 days after the operation was

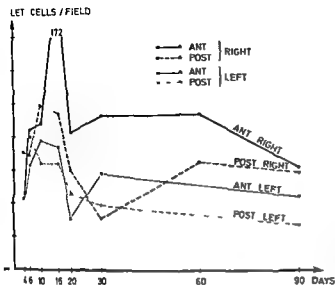


Fig 5 Interindividual median density curves representing the 12 anterior respectively the 12 posterior counting fields from each septum. Right side is closed, left side is open.

lated separately. On the occluded side there was anteriorly in the septum a significantly higher density ($p < 0.05$) than in the same segment in the normal material. On the other hand, analysis of the corresponding posterior segments showed no significant differences in density ($p > 0.05$). On the occluded side there was, during the period 20–90 days, a significantly higher density ($p < 0.04$) anteriorly than posteriorly on the same side (Fig 5).

On the open side there was no significant difference in density anteriorly ($p > 0.1$) as compared with the normal material, but posteriorly the density was significantly lower ($p < 0.025$) than at this site in the normal material. Between the anterior and posterior segments of the open side there was no significant difference in density ($p > 0.8$).

Anteriorly on the occluded side there was a significantly higher density ($p < 0.025$) than anteriorly on the open side. Posteriorly there was also a significantly higher density ($p < 0.025$) on the occluded side than in the same location on the open side (Fig 5).

DISCUSSION AND CONCLUSION

According to Hilding (1932) there ought to be a considerable increase in goblet cell density on

the occluded side and a considerable decrease on the open side. Changes in density were said to be most marked in the animal occluded longest. Our quantitative studies, however, did not show the pronounced changes reported in Hilding's publication, merely tendencies in the directions stated.

(1) A few days after the operation a considerable increase in goblet cell density occurred on both sides of the nose, but it had worn off by approx. the 20th day (Fig 4). This increase, which signifies increased differentiation of epithelial cells—presumably basal cells—can hardly be attributed to altered air flow, but rather to an irritative state caused by the operation. Below, we shall therefore consider exclusively changes in density during the period 20–90 days, the *late period* during which only changes in air flow manifest themselves.

(2) On the occluded side there was not, during this late period, any significant increase in density for the septum as a whole as compared with the normal material. A certain tendency was observed, however, there being anteriorly on the septum a significantly higher density on the occluded side than in the same location in the normal material.

(3) On the open side there was, during the

late period, a significantly lower density on the septum as a whole than in the normal material and this decrease was particularly marked in the posterior part of the septum

(4) The tendency towards higher density on the occluded and lower on the open side was more distinct, if these two sides were compared. During the late period there was a significantly higher density on the occluded side both for the septum as a whole and also when comparing only the anterior and posterior parts respectively

(5) It must be concluded, therefore, that a completely abolished air flow in the nose leads to a slight increase and doubling of the air flow to a slight decrease in goblet-cell density. The changes in density manifest themselves mainly in two locations. (A) Anteriorly on the occluded side where previously the effect of unconditioned respiratory air was at a maximum and where, accordingly, the occlusion has most effect. (B) Posteriorly on the open side, where a doubled air flow and poorer conditioning of the air will be quite particularly in evidence

In human material we have found parallels. Normal adults show anteriorly on the septum and conchae, where the mucosa is affected by unconditioned respiratory air, the lowest goblet cell density (Mogensen & Tos, 1977a, 1977b). In laryngectomized patients who do not have nasal respiration a number of authors (Sternberg 1924, Dixon et al, 1949, Naumann, 1964, Puskas et al, 1970) assessing sections of the nasal mucosa have found more numerous goblet cells than in normals. However, there is not agreement concerning the degree of goblet cell increase again emphasizing the need for accurate quantitative studies of whole mount specimens

(6) A rather interesting finding is the increase in goblet cells taking place 4 days after the operation (Fig. 5). The mechanical irritation which is at a maximum on the right septum anteriorly, gives rise to an increase in density 2 or 3 times. However there is also a question of a systemic action by the surgi-

cal procedure since goblet-cell density increased also posteriorly on the right and on the left septum

A similar reaction may be expected following surgery on the nose or larynx. A rapid increase in goblet-cell density is observed in the Eustachian tube and nasal cavity under abnormal conditions (Tos & Bakken, 1977) and must be assumed to occur in catarrhal and infectious states of the nose

(7) Determinations of goblet-cell density carried out as blind studies have shown quantitative changes in one of the main components. Other factors such as changes in ciliary cells, possible metaplastic changes in the epithelium, changes in epithelial mucosal thickness, and any processes in the mucosa propria must also be studied quantitatively

After 3 months observation the goblet-cell density on the occluded side was somewhat higher on the occluded and somewhat lower on the open side than in normal rabbits

(4) Therefore a longer observation period is needed to ascertain at which level the density will become stabilized. Moreover normalization of the air flow after re-opening the occluded side will be able to elucidate whether, and if so when, normalization of the mucosa takes place

ZUSAMMENFASSUNG

Bei Kaninchen wurde Vestibulum nasi auf einer Seite chirurgisch geschlossen. Die Tiere wurden innerhalb einer Zeit höchstens 90 Tage beobachtet. Schleimhaut der Nasensecheidewand wurde von beiden Seiten ausgewaschen nach der PAS-Alcianblau-Ganzpräparationsmethode gefärbt und die Becherzellen gezählt. Die Dichte der Becherzellendichte zwischen der geschlossenen und der offenen Seite war kleiner als es auf Grund der klinischen Angaben zu erwarten war. Totale Ausschaltung des Nasenstromes während drei Monate führte nur zu einer Vermehrung Verdopplung des Luftstromes zu einer mäßigen Verminderung der Becherzellendichte. Am Ende der ersten 16 Tage wurde eine bedeutende Verminderung der Dichte auf der geschlossenen aber auch auf der offenen Seite gefunden, die kaum als Folge der gestörten Sekretionsverhältnisse zu erklären ist.

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BACTERIOLOGY OF MAXILLARY SINUSITIS IN RELATION TO QUALITY OF THE RETAINED SECRETION

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Abstract The bacteriological findings in 200 patients with acute maxillary sinusitis are reported. It is concluded that the sampling technique—by antral aspiration—is highly significant in the evaluation of the bacteriological background of sinusitis whereas the anaerobic transport of the sample seems to be of less importance. By aspiration the purulent secretion can be properly separated from the non purulent secretion. *Pneumococci*, *H. influenzae* and anaerobic bacteria can be isolated in about 90% of the patients with true sinus empyema while sterile conditions are rare.

During the years many studies have been devoted to the bacteriology of maxillary sinusitis. Beta hemolytic streptococci, formerly a "finding in sinusitis, are seen today", sporadically while the occurrence of *Streptococcus pneumoniae* and *H. influenzae* has increased. These latter two species taken together are now isolated in 35 to 60% of the patients with acute maxillary sinusitis (Axelson & Brorson, 1972, Nylen et al., 1972, Rantanen & Arvilommi, 1973, van Cawenberge et al., 1976). Concomitantly as the clinical interest in anaerobic bacteria has increased and the anaerobic culture technique has improved, anaerobic bacteria have been isolated in sinusitis in as high a frequency as 33% or more (Frederick & Braude, 1974, van Cawenberge et al., 1976). This high incidence of strictly anaerobic bacteria in purulent sinus secretions is plausible in the light of the extremely low oxygen tension in these secretions (Carenfelt & Lundberg, 1977). So far in all reports concerned with the bacteriological background of acute maxillary sinusitis it has not been possible to demonstrate bacteria in the sinus in 15 to 35% of the patients.

With few exceptions the bacteriological analysis has been based on specimens taken from the lavage or from the nasal cavity. It has been claimed that these sampling techniques are unsuitable as the risk of contamination increases which is reflected by a high frequency of mixed flora. Cerebral irrigation also implies a dilution and a regeneration of the specimens which can result in a low density of vital bacteria in the sample taken from the lavage. When sinus was aspirated for bacteriological examination the result was not related to the quality of secretion.

The present paper reports the bacteriological findings in antral secretion obtained by aspiration from patients with a rather short history of sinusitis. Attention has been paid to the relation between bacterial isolates and the quality of the sinus secretion as well as to the influence of the transport technique used.

MATERIAL AND METHODS

Antral secretions from 200 patients with acute maxillary sinusitis and not treated with antibiotics during the previous month were examined bacteriologically.

In all cases the secretions were obtained by aspiration from patients with a rather short history of sinusitis through a Lichwitz needle inserted into the inferior nasal meatus. The secretions were classified as purulent or non purulent according to Carenfelt & Lundberg (1977).

Table I Bacterial isolates in 77 purulent and non-purulent sinus secretions transported aerobically in sputum cups

	Number of isolates	
	Purulent secretions	Non-purulent secretions
umococci	18	5
umococci & streptococci	1	
influenzae	11	1
aerobic bacteria	10	
aerobic bacteria		
streptococci	1	
aerobic bacteria		
streptococci	2	
aerobic bacteria		
epidermidis	2	
streptococci	5	
ph. aureus	3	1
growth	4	1

During the first years of the investigation 1968 to 1973 90 patients were selected for the study as having retained sinus secretion in the maxillary sinus both prior to and after 2-3 days of antibiotic treatment. The bacteriological examination was based on specimens aspirated prior to the antibiotic treatment. Only exceptionally had these patients a history of lingering sinusitis. The remaining 110 patients studied during 1974 to 1977 were selected as having retained secretion in the sinus at least at the first examination. None of these patients had a history of sinusitis of longer than one month. In patients with bilateral sinusitis the isolates of the clinically most seriously diseased sinus is reported. Thus in all the sinus secretion of 200 sinuses from the same number of patients was bacteriologically analysed.

Secretion samples collected during 1968 to 1973 were transported aerobically in sealed sputum cups without medium. During 1974 to 1977 the secretion samples were transported in the following ways. From 31 patients the aspirated secretions were transferred aerobically into agar tubes. Samples of secretion from 28 patients were injected into anaerobic broth agar bottles. From 51 patients samples of se-

cretion were transported both aerobically and anaerobically as described above. The specimens were usually managed at the laboratory within four hours and were never allowed to be stored for more than 12 hours.

Bacterial isolation

During 1968 to 1973 the specimens were cultivated on blood agar (Columbia agar base from BBL supplemented with 10% (v/v) horse blood) blood agar with 4 mg/l gentian violet endo agar (Oxoid) phenylethanol agar (Difco) supplemented with 5% (v/v) horse blood haematin agar (GC agar base supplemented with IsoVitalex enrichment and 0.2% haemoglobin BBL) and a blood agar plate incubated anaerobically in a steel jar with a cold catalyst. The blood agar with gentian violet was incubated anaerobically and the haematin agar in 5% CO₂. During 1974 to 1977 the specimens were cultivated as described above but with one exception. For isolation of anaerobic bacteria a freshly prepared blood agar plate was incubated immediately in a Gas Pak jar (BBL). The anaerobic broth agar bottles were incubated at 37°C and read daily until either growth occurred or for 10 days after which time subculturing on anaerobic blood agar plates was performed.

Haemophilus influenzae was identified on the basis of dependence on X and V factors. Pneumococci and group A streptococci were identified by their sensitivity to optochin and bacitracin respectively and serotyped by capsular reaction test and in the latter part of the study by immunoelectroosmophoresis (Wadstrom et al. 1974).

The DNase test was used to differentiate *Staphylococcus aureus* from *Staphylococcus epidermidis*. Enteric bacteria were identified using the following biochemical reactions. Production of acid and gas from mannitol hydrolysis of o-nitrophenyl D-galactopyranoside (ONPG) formation of indole urease and acetoin. *Pseudomonas aeruginosa* was identified by the oxidase reaction and the ability to grow on agar containing 0.5 g/l Cetrimide.

Table II Bacterial isolates in 54 purulent and 28 non purulent sinus secretions transported aerobically in agar tubes

	Number of isolates	
	Purulent secretions	Non purulent secretions
Pneumococci	16	7
Pneumococci <i>H influenzae</i>	1	
Pneumococci a streptococci	1	
<i>H influenzae</i>	16	2
Anaerobic bacteria	9	
Anaerobic bacteria a streptococci	3	
Anaerobic bacteria		
<i>Staph epidermidis</i>	1	
B streptococci	1	
a streptococci	2	
Branhamella catarrhalis	1	
<i>Staph aureus</i>	1	1
<i>Staph epidermidis</i>		1
<i>Pseud aeruginosa</i> Klebsiella		1
No growth	1	16

RESULTS

The bacteriological findings from 90 sinus secretions transported in sputum cups are given in Table I. In the purulent secretions predominating species was pneumococci, representing 49% of the isolates. Anaerobic bacteria alone or together with other bacteria were present in 20% of the secretions. *H influenzae* and Beta streptococci group A were isolated from 14 and 7% of the secretions, respectively. In 5% of the purulent secretions no bacterial growth could be demonstrated. Also in non purulent secretions the pneumococci were the most prevalent species found, in 5 of 13 cases. In almost half of the non purulent secretions no bacterial growth could be demonstrated.

A total of 82 samples of sinus secretion, 54 purulent and 28 non purulent were transported aerobically in agar tubes (Table II). In the purulent secretions pneumococci and *H influenzae* were almost equally common and taken together isolated in 34 of 54 secretions. Anaerobes alone or taken together with other bacteria were isolated from 15 of 54 purulent

Table III Bacterial isolates in 59 purulent and 20 non purulent sinus secretions transported in anaerobic culture bottles

	Number of isolates	
	Purulent secretions	Non purulent secretions
Pneumococci	22	6
Pneumococci <i>H influenzae</i>	1	
Pneumococci a streptococci		1
Pneumococci <i>Staph epidermidis</i>		2
<i>H influenzae</i>	14	3
<i>H influenzae</i> <i>Staph epidermidis</i>	2	
<i>H influenzae</i> <i>Pseud aeruginosa</i>	1	
Anaerobic bacteria	11	
Anaerobic bacteria B streptococci	1	
Anaerobic bacteria a streptococci	1	
Anaerobic bacteria		
<i>Staph epidermidis</i>	2	
B streptococci	1	
a streptococci	2	
Branhamella catarrhalis	1	
<i>Staph epidermidis</i>		4
<i>Pseud maltophilia</i>		1
No growth		5

secretions. Only one purulent secretion without demonstrable bacteria was found. As non purulent secretions were present the most common finding. In more than half of these secretions (16/28) no bacterial growth could be demonstrated.

Anaerobic broth agar bottles were used for the transport of 79 samples of sinus secretions, 59 purulent and 20 non purulent secretions (Table III). Essentially the same bacteriological findings were encountered as with aerobic transport used. In purulent secretions pneumococci, *H influenzae* and anaerobic bacteria made up the majority of isolates. Bacterial growth could not be demonstrated in 5 of the non purulent secretions.

When samples of purulent secretion from sinuses were transported both aerobically and anaerobically the results of the bacteriological examinations showed almost identical results for the two transport techniques (Table I).

Table IV Bacteriological findings in 37 purulent secretions aspirated from maxillary sinuses and transported aerobically and anaerobically

	Transported	
	aerobically	anaerobically
pneumococci*	12	12
<i>H. influenzae</i> *	13	14
aerobic bacteria	8	10
cellulose bacteria	4	2
growth	1	0

* One secretion pneumococci and *H. influenzae* were listed together

Considering all the 153 patients with maxillary sinus empyema from whom secretion samples were aspirated and transported aerobically, anaerobically or aerobically and anaerobically, pneumococci were present in 4%, anaerobic bacteria in 23% and *H. influenzae* in 21% of the patients

DISCUSSION

The successful treatment of maxillary sinusitis necessitates a thorough knowledge of the prevailing bacteriology. In this study, special attention was paid to the relationship between the quality of the retained secretion, purulent or non purulent, and the bacterial findings. Furthermore, interest was focused on the extent to which the bacteriological findings are influenced by the transport techniques of the specimens from sampling time to their cultivation at the laboratory

All samples of sinus secretion in the present study were obtained by aspiration through a cannula introduced through the inferior nasal meatus. By this technique the contamination of the specimens by bacteria in the nasal cavity and skin of the upper lip can be minimized as compared to specimens obtained by sinus irrigation. This is amply demonstrated in the present study by the low frequency of mixed bacterial flora and staphylococci. Another ad-

vantage of the aspiration procedure is the possibility of an accurate estimation of the severity of the sinusitis. Thus, serous secretions and purulent secretions of low viscosity can hardly be distinguished when diluted in a large volume of saline. A previous paper reported that the clinical classification of secretions as purulent and non-purulent corresponds to a clear-cut difference in bacterial and white corpuscular cell number (Carenfelt & Lundberg, 1978). In this paper the difference between purulent and non-purulent secretions is further emphasized by the fact that bacterial growth could be demonstrated in the purulent secretions from almost all the patients as opposed to the non purulent secretions. Thus, in the latter secretions lack of bacterial growth was observed in 15 to 50% of the secretions, depending on the transport technique used. This does not mean, however, that non purulent secretions are sterile. Six of 8 non purulent sinus secretions without demonstrable growth of bacteria, examined in a Bürker chamber, showed bacteria in numbers ranging between $1.1-7.3 \times 10^3/\text{ml}$. Corresponding figures for 16 purulent secretions were always $>10^3/\text{ml}$. Thus, no bacterial growth only means that the bacteria are unable to establish growth on the culture medium used.

The method of transport of bacteriological specimens has long been a subject for discussion. One possibility is to deliver the syringe with the aspirated secretion sealed by a rubber stopper, a method which preserves the anaerobic state of the purulent specimen and minimizes overgrowth of contaminating bacteria. For practical reasons other methods had to be used in this study. As opposed to samples taken with a cotton pin, a thin film of secretion and a high degree of aeration were avoided, which may explain the high frequency of anaerobic isolates. The unexpected small differences in this study between aerobically and anaerobically transported samples as regards to the distribution of bacteria support this opinion. However, overgrowth of facultative bacteria in the anaerobic broth agar

bottles may have concealed an even higher incidence of anaerobic bacteria

The frequent occurrence of pneumococci and *H. influenzae* in acute maxillary sinusitis found in this study is in accordance with most previous reports. It was recently reported that *H. influenzae* has become a more common finding in sinusitis during recent years (van Cawenberge et al., 1976). In the present study *H. influenzae* was isolated from purulent secretions in a higher frequency during the second part of the study (30–35%) as compared with the first part (14%). This discrepancy, however, does not necessarily reflect a true frequency increase of this species. The relative increase of *H. influenzae* may merely reflect a higher degree of curability in sinusitis caused by this bacteria, as in the early part of this study only patients with retained antral secretion 2 to 3 days after sample taking were selected.

In contrast to earlier studies, van Cawenberge et al. (1976) found anaerobic bacteria to be a common cause of acute sinusitis. In the present material anaerobic bacteria as a group, either alone or with various other bacteria, were the bacteria most frequently occurring in purulent secretions, next to pneumococci, which clearly indicates the importance of anaerobic bacteria as pathogens also in acute purulent sinusitis. This conclusion is further supported by the fact that anaerobic bacteria were never isolated together with pneumococci or *H. influenzae*, both well known pathogens.

As demonstrated in the present report, bacteria can almost invariably be isolated from purulent antral secretions when aspirated for examination. In contrast the isolation of bacteria from non purulent secretions is often unsuccessful, but this also depends on the method of transport of the sample. Taken together, pneumococci, *H. influenzae* and anaerobic bacteria can be isolated from the sinus in about

90% of the patients with acute maxillary sinusitis. This implies that comparatively few bacterial species have to be suspected when selecting antibiotics for the treatment.

ZUSAMMENFASSUNG

Der bakteriologische Befund bei 200 Patienten mit akuter Maxillärer Sinusitis wird hier aufgezeigt. Es ist im Schluß gezogen, daß die Technik der Punktion – durch antrale Aspiration – für die Aufklärung der bakteriologischen Grundlage von Sinusitis entscheidend ist, während der anaerobische Teil der Probe von geringerer Bedeutung zu sein scheint. Die Aspiration läßt sich das eitrige Sekret von dem eitrigen Sekret zweckmäßig trennen. Pneumococci, *H. influenzae* und anaerobische Bakterien können bei 90 % der Patienten mit echtem Sinusitis isoliert werden. Hingegen sind sterile Verhältnisse sehr selten zu treffen.

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ASPERGILLOSIS OF THE MAXILLARY SINUS

Clinical and Histopathological Features of 4 Cases and a Review of the Literature

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(Received January 5 1978)

Abstract Four patients with aspergillus maxillary sinusitis are reported and a review of the literature on aspergillus nasal sinusitis during the last 10 years is presented. The patients had symptoms and X ray findings similar to bacterial sinusitis. One patient had been operated on 6 years previously due to a chronic sinusitis and 3 patients had been treated with broad spectrum anti-fungal drugs. Caldwell Luc operations were performed to exclude malignant tumours. The aspergillus etiology of the sinusitis was discovered in routine stained sections by histo-pathological examination of the sinus mucosa. No recurrence of the fungus infection occurred after the Caldwell Luc operation which was valuable for correct diagnosis as well as being the best suited operation.

Fungus infection caused by aspergillus is a rare disorder in man in industrialized countries. The most important site of infection is the lung with or without hematogenous spread to other organs (Emmons et al, 1963, Young et al, 1970). A localized aspergillus infection also occurs in the upper respiratory tract, but is probably more common than was previously suspected (Iwamoto et al, 1972, Grigoriu et al, 1977). To our knowledge only 3 patients with sinusitis caused by aspergillus have been reported from Scandinavia (Rohr, 1964, Andersen & Stenderup 1956, Mikaelsson 1975). Consequently, and because of the frequent diagnostic difficulties, we want to report 4 additional cases.

Review of the literature

In cattle, sheep and birds, aspergillus infections are common. For example, in horses as-

pergillus is the etiologic agent in a sinusitis like condition, guttural pouch mycosis where the clinical picture is dominated by purulent discharge from the nose and invasion of blood vessels, often leading to fatal epistaxis (Cook, 1968, Bjorklund & Pålsson, 1970, Lingard et al, 1974).

In man, aspergillus infection of the maxillary sinus can behave either as an invasive or non-invasive infection (Hora, 1965). In the non-invasive infection the symptoms, clinical signs and X ray findings are similar to a chronic bacterial sinusitis. In the invasive form, bone destruction and involvement of the orbit often render the clinical differentiation from a malignant tumour difficult (Chapman & Bach, 1976).

Infections due to aspergillus in the nose and paranasal sinus, since the review of Sandison et al (1967), are summarized in Table I.

The cases reported by Sandison et al (1967) were included and reinvestigated in the articles by Milošev et al (1969) and Veress et al (1973).

Most authors report solitary cases of aspergillus infection of the paranasal sinuses, but in a recent systemic histopathological and mycological investigation, aspergillus sinusitis was seen more frequently (Grigoriu et al, 1977). In the Sudan the disease appears mostly as a granuloma with giant cell reaction, fibrosis and often involvement of the orbit (Sandison et al, 1967, Milošev et al, 1969, Veress et al, 1973). The hot climate with a low humidity, toxic

Table 1 Review of reported aspergillus paranasal sinusitis during the last 10 years

Authors	No of pats	Age and sex	Symptoms	Site	Treatment
Milošev et al (1969) Sudan	17	11 males 6 females	Proptosis Painless swelling of the ethmoid and/or maxillary region	Paranasal sinus and orbit	Medical treatment
Taillens et al (1970) Switzerland	1	F 35	Tubal obstruction with otoscleritis Chronic maxillary sinusitis	Maxillary sinus	Oral antifungal
Zinneman (1972) USA	1	M 46	Unilateral pain rapidly declining vision Mucous gineous nasal discharge Proptosis	Maxillary sinus and orbit	Amphotericin B
Iwamoto et al (1972) Japan	1	M 60	Nasal obstruction Fever Purulent rhinorrhea Check pain	Maxillary sinus	Exposure
Gngoru & Dutoit (1973) Switzerland	1	M 53	Maxillary sinusitis	Maxillary and ethmoid sinuses	Oral antifungal
Veress et al (1973) Sudan	46	25 males 21 females	Swelling of the maxillary and/or ethmoid sinuses Proptosis	Paranasal sinus and orbit	Surgery
Mikaelsen (1975) Norway	1	M 41	Cheek pain Unilat rhinorrhea Sneezing	Maxillary and ethmoid sinuses	Culture
Joch et al (1975) France	2	F 74 F 22	Unilateral nasal obstruction Purulent and hemorrhagic rhinorrhea Headache Unilateral purulent rhinorrhea Cacosomia	Paranasal sinus Maxillary sinus	Exposure and surgery Culture
Warder et al (1975) USA	3	F 67 F 63 M 18	Facial pain Nasal obstruction Hemorrhagic rhinorrhea Proptosis	Maxillary sinus Fronto-ethmoid complex	Culture Exposure
Gonty & Page (1977) USA	1	M 26	Cheek pain	Maxillary sinus bilat	Culture
Gngoru et al (1977) Switzerland	43	18 females 25 males	Nasal obstruction Rhinorrhea Orbital pain Headache	Maxillary sinus	Exposure and surgery Culture Leucine

imen logy	Mycology	Remarks
ranuloma rate hyphae cells	<i>A. flavus</i> cultured	
-green anch ng	<i>A. niger</i> cultured	
us tissue inflamm muco- hyphae inter s Mucor	<i>A. flavus</i> and <i>Nocar dia asteroides</i> cultured	Cutaneous fistulae in the ca nine fossa
wn mass tes assumed to rgilus	-	
mass m suggested hus	<i>A. fumigatus</i> cultured	
c grey te mass tubercles with necrosis hyphae and in tory cells with ls	<i>A. flavus</i> cultured	Prolifera tive form Exudative necrotiz ing form Mixed form
ee lium o Aspergillus	-	
f id o. <i>Aspergillus</i> mycelia	-	Immunor el phoresis <i>A. nidulans</i>
brown foreign body ma. <i>Aspergil a</i>	<i>A. flavus</i> cultured	Tooth ex traction 6 months previously
thickening lent necrotic lloma	-	
tooth ball of hyphae	<i>A. fumigatus</i> cultured	Op max frac ture 6 years previously Caldwell Luc 2 years pre viously due to chron max
inflamed mu th mycelia	<i>A. fumigatus</i> (40) <i>A. niger</i> (2) <i>A. flavus</i> (1) cultured	Mucopuro lent form Budding and/or ca seous form Pseudotu mor form

metabolites of the fungus and the local immunological conditions are discussed as a possible explanation for this concentration of the disease in the Sudan (Sandison et al , 1967, Veress et al , 1973) For us, another possible explanation could be the living habits and the environmental conditions in parts of the Sudan, where among the Nuer, for instance, the carcasses and bodily products of the cattle are widely used for household purposes. Cattle dung is used for plastering walls and floors of straw huts in cattle compounds, as plaster to protect wounds and as tooth powder (Evans-Pritchard, 1940) From the report by Milošev et al (1969) it is also evident that many of the patients were working as farmers and aspergillus species were isolated from the environment of the patients (Sandison et al , 1967)

CASE REPORTS

The clinical features of the 4 cases all with left sided involvement of the maxillary sinus studied in the present investigation are summarized in Table II

There was a considerable age variation in the material consisting of 1 female and 3 male patients. The female had a previous history of Caldwell Luc operation 6 years earlier due to a chronic maxillary sinusitis after a tooth extraction with sinus perforation. The 3 male patients had been in good health. None of the patients suffered from a malignant or systemic disease. The duration of symptoms before the present operation varied markedly between the patients. One patient (M 80) had symptoms of bad smell from the nose for 3 years. The first symptom of the disease in one patient (M 61) was an acute attack of epistaxis. Sinus X ray revealed mucosal swelling or opacification. All 3 male patients had been treated with tetracycline for their sinusitis. Due to persistent symptoms and in spite of a regime of nasal decongestant, antibiotics and lavage all 4 patients were operated *ad modum* Caldwell Luc. The mucosal lining of the sinuses were found to be edematous and markedly thickened. In 2 of the patients greyish necrotic masses were observed and in one of these (M 80) the medial sinus wall was replaced by necrotic tissue.

Histo-pathology

Microscopic examination of the material removed at Caldwell Luc operation revealed an inflamed respiratory mucosa diffusely invaded by numerous polymorphonuclear granulocytes and mononuclear inflammatory cells. Eosinophilic granulocytes were not very conspicuous. Separated from or situated on the surface of the pseudostratified ciliated columnar epithelium, separate and

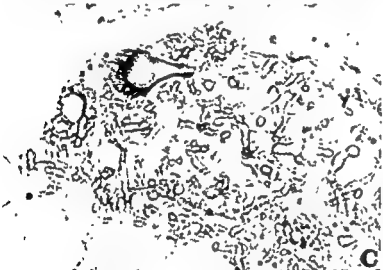


Fig. 1a. Micrograph of inflamed maxillary sinus mucosa with adjacent, partly necrotic fungal mycelia. Hematoxylin-eosin, $\times 40$.

Fig. 1b. Maxillary mucosa with suspicion of fungal invasion. Hematoxylin-eosin, $\times 100$.

Fig. 1c. Fungal mycelia showing dichotomous branching. Hematoxylin-eosin, $\times 400$.

dichotomous branching hyphae with conidiophores were observed (Fig. 1a). Occasionally the mycelia were suspected of infiltrating the mucosa, as depicted in Fig. 1b. No granuloma or giant cell reaction with fibrosis could be detected. The pigmented conidiophores ended in a gradually widening vesicle (Fig. 1c). The upper part of the vesicle (capitulum) was covered with a single layer of parallel arranged primary sterigmata. Spherical conidia, sometimes forming unbranched chains, were observed in the vicinity of the fruiting bodies (Fig. 1d) (Thom & Raper, 1945). No cultures were performed but the morphology of the fungus was characteristic of *Aspergillus*.

et al., 1963). The diagnosis of the mycotic infection could be made in sections stained with hematoxylin-eosin or

according to van Gieson. Staining with Green, Grid and PAS are not necessary for the histopathological diagnosis.

DISCUSSION

Aspergillus infection of the maxillary sinus can be found in patients in general good health or in nutritionally deprived persons and in patients with an underlying debilitating disease (Emmons et al., 1963). Systemic diseases, such as collagenosis and malignant tumours, and also treatment with immunosuppressive drugs and cytostatic agents, have been associated with

Table II Cases of aspergillosis of the maxillary sinus

Case	Predisposing factors	Symptoms	X ray	Treatment	Follow up
1	Chronic max. sinusitis after tooth extraction and Caldwell Luc op 6 years previously	Cheek pain nasal discharge Rhinorrhea	Opacification	Caldwell Luc	Control after 3 months and telephone contact after 3 years no symptoms
2	Tetracycline treatment	Cheek pain Epistaxis	Opacification	Caldwell Luc	Control after 9 months and telephone contact after 5.5 years no symptoms
3	Tetracycline treatment	Unilateral nasal discharge Cacosoma	Mucosal thickening Fluid	Caldwell Luc	Control after 1 month no symptoms
4	Tetracycline treatment	Cacosoma	Opacification	Caldwell Luc	Control after 3 months no symptoms

increased incidence of fungal infections (Jung et al., 1970). In patients in general good health there are probably some factors, systemic or local, predisposing to the fungal infection of the paranasal sinus (Chapman & Smith 1976). Most patients reported have been treated with broad spectrum antibiotics, which suppress the bacterial flora and support a fungal overgrowth. Recurrent bacterial infection after tooth extraction trauma or surgery may affect the drainage of the sinus.

Our 4 patients were all in good health, except for their ENT symptoms. Three of the patients had been treated with lavage and systemic tetracycline drugs without improvement of the disease prior to operation. One patient (M36) had 6 years previously been operated *ad idem* Caldwell Luc for a sinus infection after a tooth extraction. In all patients the aspergillus infections were of the non invasive type except for the oldest patient (M80) where the medial wall of the maxillary sinus was destroyed and at operation found to be replaced by a "necrotic granular mass". Histopathological examination of the tissue removed from this patient revealed a suspicious fungus infiltration in the mucosa. However, this elderly patient did not suffer from any debilitating disease or nutritional deficit, but the systemic involution and cellular immune dysfunction known to occur in elderly persons

could have caused decreased host defence and enhanced a destructive infection.

In none of the patients was the mycotic infection suspected preoperatively, and no cultures with respect to fungus were performed. The etiology of the sinusitis was obvious first upon histo-pathological examination of the surgically removed material. Due to the mycelial structure and the presence of fruiting bodies the diagnosis of aspergillus infection could be made on routine stained sections. The Caldwell Luc operations have been of diagnostic importance as well as the therapy of choice, as not all infections of the paranasal sinuses can be satisfactorily treated by lavage and antibiotics. No other treatments were given after the operations and all patients were free from symptoms at follow-up, indicating that with Caldwell Luc operation no additional local or systemic drug therapy is necessary.

The clinical conclusions from the review and our reported cases are (1) A maxillary sinusitis which is not improved by lavage and oral antibiotic therapy should be subjected to diagnostic Caldwell Luc operation. (2) The diagnosis of aspergillus infection can be established by histo-pathological examination of routinely stained sections. (3) The Caldwell Luc operation is also the therapy of choice in cases of aspergillus maxillary sinus infection.

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ZUSAMMENFASSUNG

Vier Patienten mit Aspergillusinfektion der maxillaren Nebenhöhle und eine Literaturübersicht von Aspergillusinfektionen der Nasennebenhöhlen der letzten 10 Jahre werden präsentiert. Symptome und Röntgenbefunde der Patienten sind ähnlich einer chronisch bakteriellen Sinusitis. Ein Patient wurde vor 6 Jahren wegen einer chronischen Sinusitis operiert und drei Patienten hatten Behandlung mit Breitspektrumantibiotika bekommen. Operation ad modum Caldwell-Luc, auch um einen malignen Tumor auszuschließen, wurde bei allen Patienten durchgeführt. Die Aspergillusätiologie der Sinusitis war bei der mikroskopischen Routineuntersuchung der Sinusmucosae erfaßt. Kein Rezidiv der Pilzinfektion konnte nach der Operation festgestellt werden. Die Caldwell-Luc-Operation brachte die korrekte Diagnose und war gleichzeitig die adäquate Therapie.

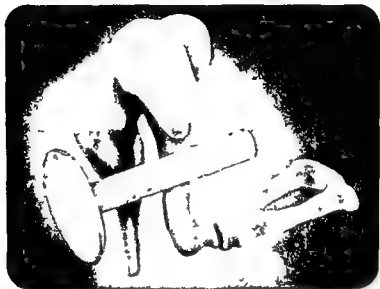
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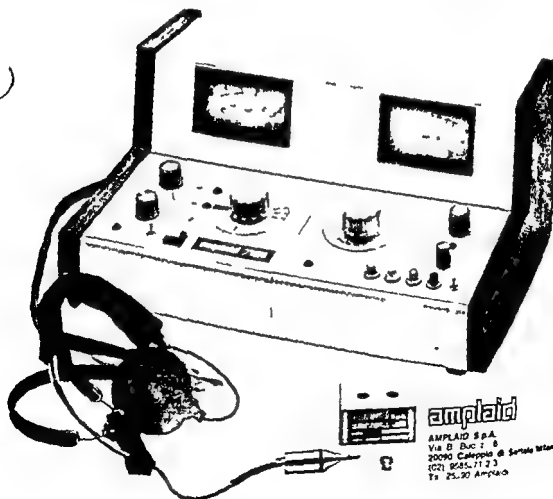
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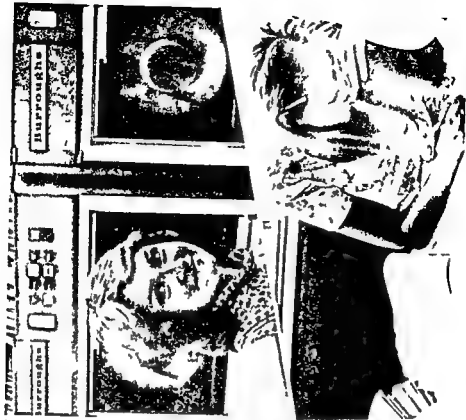
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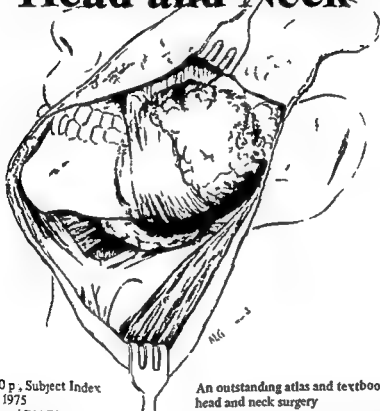
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THE EFFERENT INNERVATION IN THE REGION OF INNER HAIR CELLS IN THE ORGAN OF CORTI

W Fırbas

From the First Institute of Anatomy, University of Vienna, Vienna, Austria

(Received March 7, 1978)

Abstract The efferent innervation of the inner hair cells of the organ of the laboratory rat and of a bat was investigated with help of histochemical staining of the inner spiral bundle. The area of acetylcholinesterase-positive fibres in the inner spiral bundle regions was measured. The bulk of the efferent fibres lie in the middle part of the cochlea. Towards the apical and lower basal turns a marked decrease in the number of fibres was observed.

In contrast to the data available on the efferent innervation in the region of outer hair cells there is little data available on it in the inner hair cells. By means of a histochemical method and staining an attempt was made to obtain a general view of efferent fibres in the inner hair cell region. This study was suggested by Professor Klinke, Frankfurt. Competent structures of efferent innervation in this area are, first and foremost, the region of the inner spiral bundle (inner spiral plexus), the spiral tunnel bundle, and direct efferent endings at inner hair cells. Efferent innervation is functionally effective either in the receptors or the afferent fibres originating there. This study is especially concerned with the inner spiral bundle.

MATERIAL AND METHOD

The efferent fibres in this region were marked with acetylcholinesterase (AChE). The preparations of AChF were made by incubating the previously fixed and frozen sections of rats and two species of bats (*Microchiroptera*) according to Karnovsky's method. The incubation periods were generally long, from 24

hours) In this way the positively reacting fibres were made quite distinct. The preparations were evaluated particularly with regard to the arrangement of the efferent fibres in the individual parts of the cochlea. The diameters of the precipitate planes were measured, and the areas calculated from these diameters using the circle or ellipse formula (Fig. 1). The area was taken to be an indication of the innervation density.

RESULTS

Before considering the results, a brief discussion of the structure and composition of the inner spiral bundle (ISB) is necessary. According to investigations made by Smith (1967), Smith & Takasaka (1971), Spoendlin (1973, 1975) and others, the inner spiral bundle consists of a network of predominantly efferent fibres which are closely connected with afferent dendrites. These afferent dendrites originate mainly from the inner hair cells. Besides the efferent fibres, some afferent spiral neurons run within the ISB (Spoendlin, 1969, Sobkowicz et al., 1975, Perkins & Morest, 1975). According to the investigation of Wright & Preston (1975) the ISB and the spiral tunnel bundle have a close structural relationship. In our study we were not able to evaluate the spiral tunnel bundle.

The place of origin of efferent fibres and how they form synapses with other structures seems to be of special significance for the explanation of our own findings. The origin of

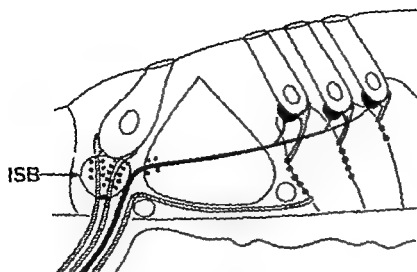


Fig. 1 Innervation of Cochlea (after Spoendlin 1973). The inner spiral bundle (ISB) is surrounded with a circular line

the efferent fibres in the ISB was investigated by experimental severing of those fibres in various mammals. Table I shows some of these findings. From them we can see that, depending to a certain degree on species and part of the cochlea, a greater or lesser number

of ISB fibres originate from the uncrossed olivocochlear bundle (UOCB). When transecting the crossed olivocochlear bundle (COCB) it appears that the main part of the efferent fibres in the ISB (inner spiral bundle) of the rat stems from the uncrossed OCB as already described by Iurato (1964). From our own experiments, midline brain stem lesions in rats showed that the COCB runs to the outer hair cells and to the basal part of the ISB. The apical ISB on the other hand comes mainly from the UOCB. This is also in agreement with the results of Warr (1975), who found that 60% of the efferent fibres in the kitten originated from the ipsilateral side. Ross et al. (1977), however, have doubts about the Warr's method: the uptake of horseradish peroxidase does not seem to be specific for the efferent olivocochlear neurons in the brainstem.

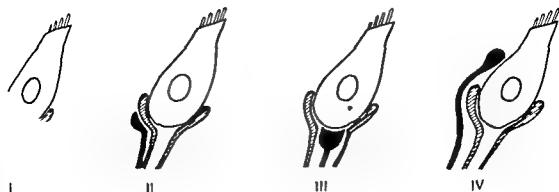
The second point of importance for evaluation is the formation of synaptic structures which have been identified in various locations in connection with the efferent fibres of the ISB. In our own preparations for light microscopy, the existence of axosomatic structures

could of course not be directly verified from the precipitates. A comparison of the preparations suggests that with rats the precipitates are nearer to the inner hair cells than with the bats.

Species dependency in connection with axodendritic synapses has already been described with regard to electron microscopic investigations. Angelborg & Engstrom (1973) and Spoendlin (1966) point to the presence of axosomatic synapses in rodents, whereas practically only axodendritic synapses could be identified in other mammals. Figure 2 shows diagrams of the various findings. Diagrams I and II illustrate the varying positions of the axodendritic synapses, diagrams III and IV show those of the axosomatic synapses. It is to be postulated according to Spoendlin with the cat (1973), II according to Smith (1967) III according to Angelborg & Engstrom with the

Table I Origin of efferent fibres in the inner spiral bundle

Species	COCB	UOCB
Squirrel monkey (Nakai & Igarashi 1974)	70% basal 50% basal	~ 40%
Cat (Spoendlin 1973)	-	100%
Chinchilla (Iurato et al 1968)	-	100%
Guinea pig (Wright & Preston 1973)	+	+



2 Efferent synaptic structures (black) on inner hair

ne pig (1973), and IV according to Wright Preston (1973) and according to the SEM of Bredberg in the rabbit (1976). A third point, which has to be considered the explanation of our histochemical findings concerns the sediment areas of the reaction products. Ultrahistochemically, acetylcholinesterase activity is identified in the axoplasmic membranes of the efferent fibres and in their presynaptic expansions (Iurato et al., 1971). The light microscope can only give a rough view of the distribution of the ferment. The area of the sediment, however, could be determined relatively precisely, and measurements yielded identical results for the individual section series. In accordance with the work of Spoendlin (1970), who found widely varying quantities of internal spiral fibres in the basal turns of cats, our own tests produced widely varying diameters of the precipitate in the basal turn. Table II gives the results of the precipitate planes in the region of the inner spiral bundle in the individual turns. The figures for the areas (in μm^2) were obtained by an average value calculation for the individual turns of 5 rats and three specimens of *Barbastella barbastellus*. We found a low degree of ferment sedimentation in the ISB in the basal and apical turns. The lowest value was measured in the basal turn (hook part) at 6 μm^2 , the smallest area in the apical part amounted to 14 μm^2 . Because of their size, the results yielded lower values. In the case of the

bat a more pronounced decrease, both apically and basally, could be noted in the efferent fibres of the outer hair cells, than was the case in the efferent fibres in the ISB, as far as the histochemical preparations could demonstrate. Perkins, in 1973, described a stronger efferent control in the region of the inner hair cells of cats and rats by using Golgi preparations.

DISCUSSION

The present study attempts to evaluate the efferent fibres of the ISB in the individual cochlear turns, by using histochemical methods. Hitherto the only available findings were those of Hilding & Wersäll (1962) and of Ishii & Balogh (1968). Hilding & Wersäll described an apically weaker reaction in the ISB of guinea pigs, whereas Ishii & Balogh speak of the same reaction strength in all turns. As postulated by Klinke & Galley (1974) efferent innervation in the region of the inner hair cells

Table II Section areas (mean values in μm^2) of the inner spiral bundle in the different parts of the cochlea

Turn	Rat	Bat
Lower basal	61	11
Upper basal	196	35
Middle	163	42
Apical	72	28

does not seem to be the same in all turns. The basal and apical decrease corresponds to the decrease in the efferent innervation in the outer hair cells, although not in every respect (Ishii & Balogh, 1968; Spoendlin, 1969). Fex (1973) postulated control mechanisms, which influence the outer and inner hair cells in the same way in order to explain the findings of Wiederhold & Kiang (1970). In this connection, however, it is necessary to explain how much influence crossed and uncrossed efferent fibres have in the formation of the ISB in the individual species.

The present study in the rat led to the same result as was obtained by Wright (1975) in the guinea pig with a silver impregnation technique. The greater part of the ISB lies in the middle cochlea. The bundle is markedly reduced in size in the apical and lower basal turns. Fex & Wenthold (1976) measured the choline acetyltransferase activity in the guinea pig cochlea. The activity was higher in the 4th turn than in the fourth turn and was lower, when the region of the inner hair cells was included in the preparations. These findings support the hypothesis that the olivocochlear fibres are cholinergic and also that in the middle part of the cochlea the inner hair cells have a stronger efferent innervation.

ZUSAMMENFASSUNG

Die efferente Innervation der inneren Haarzellen im Cortischen Organ wurde mit Hilfe der Darstellung Acetylcholinesterase positiver Fasern im inneren Spiralbündel der Ratte und einer Fledermausart untersucht. Es ergab sich gemessen an der Fläche der Fermentablagerungen im mittleren Teil der Cochlea die stärkste efferente Innervation der inneren Haarzellen. Gegen die Spitze und besonders gegen die untere Basalwindung nimmt die Zahl der efferenten Fasern im inneren Spiralbündel deutlich ab. Der Ursprung der Fasern im inneren Spiralbündel der Ratte liegt für den apikalen Teil der Cochlea vorwiegend im ungekreuzten olivocochlearen Bündel. Die Wege der efferenten Innervation im Gebiet der inneren Haarzellen (direkte Synapsen an den Haarzellen, der Synapsen an den afferenten Dendriten) werden diskutiert. Die Ergebnisse der vorliegenden Untersuchung stehen mit den Resultaten anderer histologischer und biochemischer Untersuchungen in Einklang.

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GLYCOGEN ACCUMULATION IN REISSNER'S MEMBRANE FOLLOWING CHEMICAL SYMPATHECTOMY WITH 6-HYDROXYDOPAMINE

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Abstract Rats were chemically sympathectomized by injecting them with 6-hydroxydopamine repeatedly during early postnatal development. Litter mates left untreated or injected with the vehicle only were used as controls. Inner ear tissues from a series of treated and control rats 11-72 days of age were fixed, then microdissected and prepared by usual methods for transmission electron microscopic study. Control rats 11 days of age showed accumulation of glycogen particles in Reissner's membrane particularly in the epithelial cell layer near its attachment to the spiral limbus. The epithelial cells in that region also had rough endoplasmic reticulum with greatly widened cisternae containing a granular substance. The glycogen was not present after 11 days and the cisternae diminished in size with further postnatal development. The epithelial cells of the membrane in controls of all ages had intercellular clefts that were closed at the endolymphatic surface by zonulae occludentes, open narrowly at their basal end and variously dilated in between. Coated vesicles were common at apical and lateral cell borders as well as intracellularly. Treated rats, on the other hand, showed an accumulation of glycogen particles in the epithelial cells that increased between 11 and 32 days and persisted in some cells at 72 days. The glycogen particles β in type were electron dense, ~ 150 - 300 Å in diameter and often aggregated in large masses, particularly in the lateral cell regions. Concomitantly with glycogen accumulation, coated vesicles diminished numerically and the intercellular clefts narrowed, becoming of uniformly narrow dimensions by 32 days. An upturn in the number of coated vesicles and a widening of the intercellular clefts occurred by 72 days. The results are interpreted as indicating that the epithelial cells of Reissner's membrane have an energy-dependent, coupled ion/water transport capability that is ordinarily supported by glucose metabolism. It appears that the glucose metabolism is in turn regulated by catecholamines, suggesting the existence of β adrenergic receptors on the epithelial cells of Reissner's membrane.

Experimental manipulation of the adrenergic nervous system of the rat by use of 6-hydroxydopamine, a substance known to induce

chemical sympathectomy under appropriate conditions (Tranzer & Thoenen, 1967, 1968; Thoenen & Tranzer, 1968; Malmfors & Sachs, 1968, and others), was carried out in order to learn whether or not sites of catecholaminergic influence on inner ear function would be revealed. Because the inner ear is normally endowed with a rich supply of adrenergic nerve fibers, as first documented by Spoendlin & Lichtensteiger (1966), it was expected that the absence of circulating catecholamines might cause ultrastructural changes in special target cells, or signs of abnormal development or of deterioration of the organ of Corti. It seemed highly unlikely that so abundant an adrenergic innervation could be without importance for the inner ear, although convincing experimental evidence of the functional significance of its sympathetic nerve supply is still lacking.

It is well recognized that catecholamines are involved in the regulation of glycogen metabolism in other regions of the body. Thus, for only two cochlear tissues have been identified as storage sites of glycogen, the stria vascularis and the organ of Corti, on the basis of histochemical (Belanger, 1957; Vinnikov, Titova, 1957, 1964; Falbe-Hansen, 1963, 1964; Falbe-Hansen & Thomsen, 1963) and more recent chemical (Matschinsky & Thalmann, 1971; Thalmann, 1971, 1975) determinations. β -adrenergic tissues also store glucose (Matschinsky & Thalmann, 1970; Thalmann, 1971; Thalmann et al., 1972).

Only rarely has glycogen been described in cochlear cells at the ultrastructural level. This may be due in part to the difficulty often encountered in preserving and staining glycogen unless certain procedures are followed (Revel et al., 1960, de Bruijn, 1973). Hilding et al. (1977), utilizing a method recommended by de Bruijn, documented the onset and course of glycogen storage in the developing rat cochlea. They found that glycogen appeared in abundance first in Reissner's membrane in the fetus (day 17), then in the pillar cells and the stria vascularis, disappearing from these areas by 30 days after birth. In the adult rat, glycogen occurred in a finely dispersed state in the outer hair cells with no detectable gradient from base to apex other than could be accounted for on the basis of cell size.

No previous histochemical, microchemical or ultrastructural findings have suggested that glycogen metabolism or storage occur in Reissner's membrane except as a relatively brief developmental phenomenon. This report deals with ultrastructural evidence of extensive glycogen accumulation by the epithelial cells of Reissner's membrane after chemical sympathectomy with 6-hydroxydopamine, with concomitant changes in the numbers of vesicles and the size of the intercellular spaces.

MATERIAL AND METHODS

Thirty-two Sprague-Dawley rats were injected with 6-hydroxydopamine (50 $\mu\text{g/kg}$ body weight) according to one of the two following schedules. One group of 21 rats was injected on the day of birth, then every other day until a total of 4 doses was given (last dose on day 7). Another group of 11 rats was injected on day one, then every other day for seven doses, followed by an injection every fifth day until day 30 or sacrifice, whichever came first. Eleven litter mates were used as controls, 8 of these rats were left untreated and 3 were injected with the vehicle only (ascorbic acid in physiological saline). The purpose of the mul-

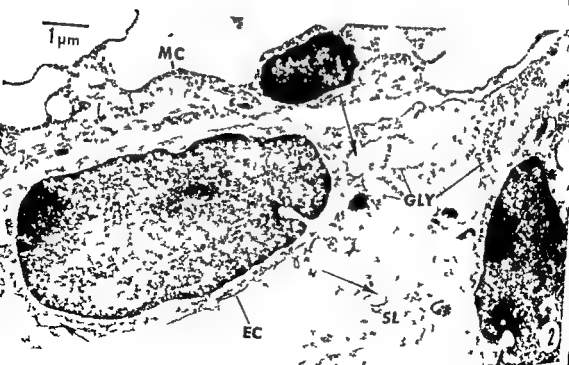
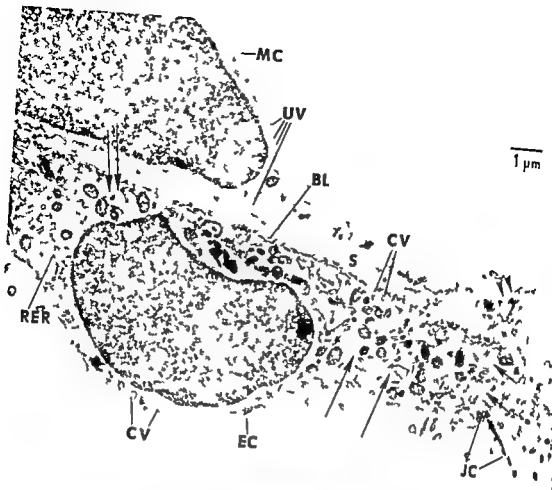
tipole injections was two-fold to induce as complete a sympathectomy as possible and to guard against the possibility of regeneration of functional adrenergic terminals for a significant period of time.

A series of inner ears from treated and control rats was obtained by sacrificing animals on days 11, 17, 22, 28, 32, and 72 after birth. Animals were first anesthetized with chloral hydrate, then perfused systemically by the intracardiac route with a mixture of 3% glutaraldehyde, 4% paraformaldehyde in Millonig buffer (pH 7.35). Next, the cochleas were dissected in additional fixative and opened to allow better penetration of fluids. The tissue was then decalcified in EDTA glutaraldehyde (Baird et al., 1967) and post fixed in 1% osmium tetroxide in Millonig buffer. The tissues were dehydrated and embedded in Epon according to routine procedures.

Eight treated rats covering the time periods noted above were chosen at random and used in this report. Five litter mates were studied as controls. The embedded tissues from these rats were thick-sectioned and stained with toluidine blue for orientation purposes. The blocks were trimmed, thin sectioned, and the sections were mounted on grids, stained with uranyl acetate and lead citrate (Reynolds, 1963), and studied in a Siemens Elmiskop 10A transmission electron microscope.

Over-t signs of chemical sympathectomy

All treated animals were observed for physiological and/or behavioral changes that might be indicative of chemical sympathectomy. Treated rats demonstrated certain signs of autonomic imbalance that varied in severity from one animal to another. The most frequently observed signs included excessive salivation, frequent defecation, and dribbling of urine. After mild stimulation, the treated young animals often fell asleep while control animals returned to grooming and exploratory behavior. The most surprising effect was that



ated animals were often difficult to anesthetize requiring two or more times the amount of anesthetic necessary for controls

RESULTS

Control Animals

The general ultrastructure of Reissner's membrane is well known and need not be described here. Certain details are presented, however, in order to consider fully the changes which occurred in the membranes of animals treated with 6-hydroxydopamine.

Mesothelial cells

In control animals, the mesothelial cells of Reissner's membrane were thin and sheet-like except for the region of the nucleus which projected into the perilymphatic space (Fig. 1).

The typical two-layered structure of Reissner's membrane is shown in this electron micrograph. The epithelial cells (EC) are attached to one another apically by a junctional complex (JC) often consisting of a zonula occludens, a zonula adherens, and a macula adherens (desmosome). A short distance below this complex, the inter-

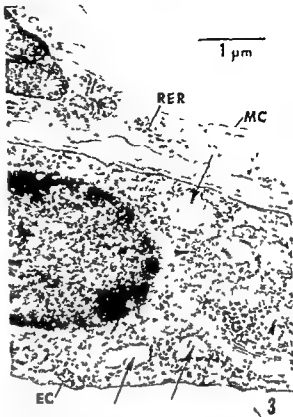


Fig. 3. A portion of normal Reissner's membrane is more highly magnified here to better demonstrate the widely dilated cisternae of rough endoplasmic reticulum (arrows) filled with a granular substance typically found in the epithelial cells (EC) at the juncture with the spiral limbus in very young rats. A portion of the Golgi complex (G) and large patches of glycogen particles (arrowheads) are present in the area of these cisternae. The relative size of the cisternae can be determined by comparison with the more typical rough endoplasmic reticulum (RER) in the mesothelial cells (MC) above. 11-day-old normal rat.

are common at all cell border membranes. The mesothelial cells are bounded basally by a basal lamina. Between this lamina and the undulating basal border of the mesothelial cells (MC) is a space of varying thickness containing a small amount of delicate, filamentous material (F). The mesothelial cells often overlap one another (arrowheads) but junctional complexes are lacking. The mesothelial cells contain the usual types of organelles although they are scantier than in the epithelial cells. Numerous uncoated vesicles (UV) are seen at all boundary membranes. The bar indicates 1 μm on this and following electron micrographs. 28-day-old normal rat.

2. The region of the junction of Reissner's membrane with the limbus is modified in young animals. The epithelial cells (EC) of Reissner's membrane contain rough endoplasmic reticulum with greatly widened cisternae filled with a granular material (arrows). This same rough endoplasmic reticulum is found in the first few epithelial cells continuing over the spiral limbus. Glycogen patches (GLY) are common in the epithelial cells of the junction region. Some of the epithelial cells of the limbus have cytoplasm that stains dark with osmium (D to the right). MC, mesothelial cell. 11-day-old normal rat.

The cells sometimes closely overlapped one another at their borders, but no junctional complexes occluded the free diffusion of perilymph around and between them. Because of the general thinness of the cytoplasm and the absence of occluding intercellular junctional complexes, it was remarkable that numerous vesicles were present at the boundary membranes and intracellularly. Most of these vesicles were uncoated, although a few coated vesicles were observed.



Fig 4 Eleven day old treated rats show large patches of glycogen (double arrows) in some epithelial cells of Reissner's membrane particularly near the junction with the spiral limbus (SL) as illustrated here. The cisternae of the rough endoplasmic reticulum of the epithelial cells are widely dilated and filled with a granular substance (single arrows). The cell (D) in the center of the field has

more dense cytoplasm that stained dark with osmium. basal lamina G Golgi complex MC mesoepithelial cell. 11 day old treated rat 4 doses 6-hydroxydopamine. Fig 5 The glycogen accumulates in aggregates (double arrows) in many of the epithelial cells of Reissner's membrane in treated rats by 17 days. The aggregates are especially common in the lateral cell regions where the

The basal surfaces of the mesothelial cells are irregular but the apical borders were generally smooth. The depth of the basal undulations largely determined the width of the fluid compartment separating mesothelial and epithelial cell layers. This compartment, which contained a small amount of delicate filamentous material, was confluent with the perinymph of scala vestibuli through the intercellular spaces.

Epithelial cells

Epithelial cells had abundant cytoplasm and were of rather uniform thickness except in the nuclear region where the cells projected slightly into the endolymphatic space (Fig. 1). These cells contained much rough endoplasmic reticulum and numerous mitochondria, especially in the regions close to the intercellular clefts. The Golgi complex was extensive, reaching from the perinuclear regions of each cell into the matrix near the clefts. Coated vesicles were commonly observed at the endolymphatic surface of the cells and intracellularly, particularly near the lateral borders. Numerous microvilli were present along the endolymphatic surface of each epithelial cell. The lateral cell margins were thrown into complicated plicae, increasing the surface area of the cells enormously and causing the formation of intercellular clefts of widely varying dimensions (Fig. 1). Each cleft was closed at the endolymphatic lumen by a zonula occludens and a zonula adherens and then a desmosome commonly followed to complete the junctional complex. The clefts were narrow at their basal outlets and covered only by the continuous basal lamina. Between the apical and the basal ends, the intercellular clefts were regular and dilatations were common.

As in the case of the organelles commonly found there, the intercellular clefts are variously dilated at 17 days (single rows) with some narrowing evident. Coated vesicles are obvious at the endolymphatic surface of the epithelial cells (EC) as shown here but are uncommon elsewhere. MC, mesothelial cell. 17-day-old treated rat does 6-hydroxydopamine.

The basal surfaces of the cells often showed complex, plicated invaginations (Fig. 1). The mouths of the invaginations were narrow and morphologically comparable to the basal outlets of the intercellular clefts.

The juncture region of Reissner's membrane with the limbus showed certain morphological modifications in young and weanling rats (Fig. 2) that tended to disappear by 28 days. The epithelial cells of this region had rough endoplasmic reticulum with greatly widened cristae containing a somewhat granular (but otherwise amorphous) substance (Figs. 2 & 3). This same type of rough endoplasmic reticulum was present in the next few cells which extended over the surface of the spiral limbus. Some of the latter cells had cytoplasm that stained dark with osmium. Lysosomal bodies were common and the abundant mitochondria tended to be large.

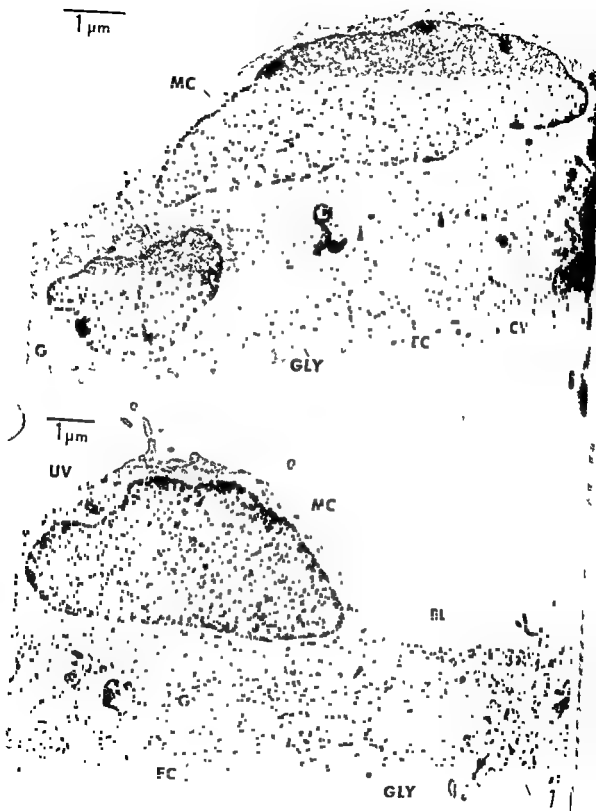
Glycogen particles $\sim 150\text{--}300\text{ \AA}$ in diameter were present in both cell layers of Reissner's membrane at its junction with the spiral limbus in 11-day-old rats, but not in the older control animals. The particles, which were electron dense, were more numerous in the epithelial cell layer. Sometimes, the accumulations of the polysaccharide particles were large (Fig. 3). Occasionally, small clusters of the particles in the aggregates appeared to be confined within membranous structures, or remnants of them, that seemed collapsed or shrunken leaving clear zones in the surrounding matrix.

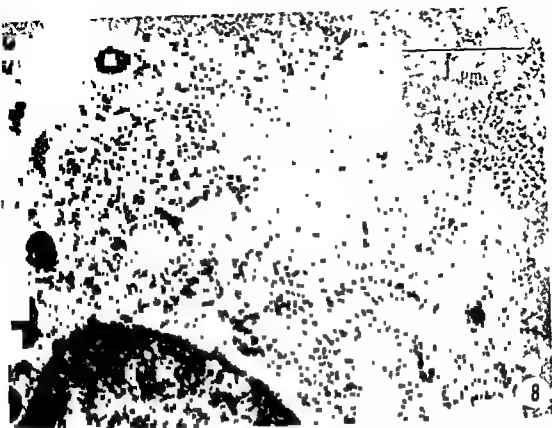
6-Hydroxydopamine Treated Rats

Reissner's membrane showed certain changes with respect to cytoplasmic inclusions or granules and intercellular spaces in treated animals 11–32 days of age. These changes were more pronounced in the epithelial than in the mesothelial layer and tended to diminish with further time.

Mesothelial cells

There appeared to be a decrease in the number of vesicles in treated rats up to and including





8 Large amounts of glycogen remain in some of epithelial cells of Reissner's membrane even at 72 days. The glycogen particles are of the β type, as shown at higher magnification and are sometimes in linear string like arrangements (examples indicated by arrows).

Many of the glycogen particles are found in the territory of the Golgi complex (G) and numerous mitochondria are nearby (M). A nucleus 72-day-old treated rat 12 doses 6-hydroxydopamine.

days of age compared with controls (Figs 5, 6 & 7). Vesicles were, nevertheless, still

6 Glycogen aggregates (GLY) almost fill the epithelial cells of the 32-day-old treated rat as shown here. Coated vesicles (CV) are found in numbers only at the doliymphatic surface. Lysosomes (L) are numerous in intercellular clefts are uniformly narrow (single arrow) and the space between the epithelial (EC) and mesothelial (MC) cells is almost obliterated. G Golgi complex 32-day-old treated rat 4 doses 6-hydroxydopamine.

7 Some of the glycogen in the epithelial cell (EC) shown here is in aggregates (GLY) but elsewhere the particles are more dispersed. The intercellular clefts are narrow (single arrow). The basal lamina (BL) is distinguishable but the space between the two layers of Reissner's membrane is much diminished in width. Uncoated vesicles (UV) are numerous in the mesothelial cell (MC) shown here. G Golgi complex 32-day old treated rat 4 doses 6-hydroxydopamine.

numerous. In contrast, the number of lysosomes increased. Small patches of glycogen occasionally occurred in the cytoplasmic matrix. The polysaccharide particles were variable in size (~ 150 – 300 Å in diameter) and electron dense. Additionally, the basal surfaces of the cells showed fewer pronounced undulations during the early post natal period, the space between the mesothelial and epithelial cell layers was all but obliterated by 32 days (Figs 6 & 7). Except for the persistence of occasional small patches of glycogen (Fig 8), the mesothelial cells distal to the limbus junction region resumed a more typical overall appearance by 72 days (Fig 9).

The mesothelial cells near the junction with the spiral limbus generally contained some gly-

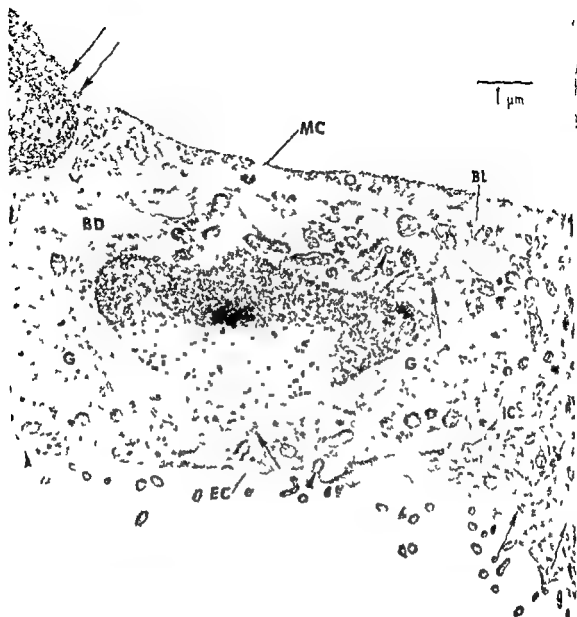


Fig 9 Variation in the amount of glycogen present occurred from one site to another in Reissner's membrane at 72 days. The epithelial cell (EC) in the center of the field with more clear cytoplasm has widely dispersed small patches of glycogen particles (arrows) whereas the cell with more electron dense cytoplasm at lower right has slightly larger patches. Glycogen particles (arrows) are also present in the mesothelial cell (MC). The intercellu-

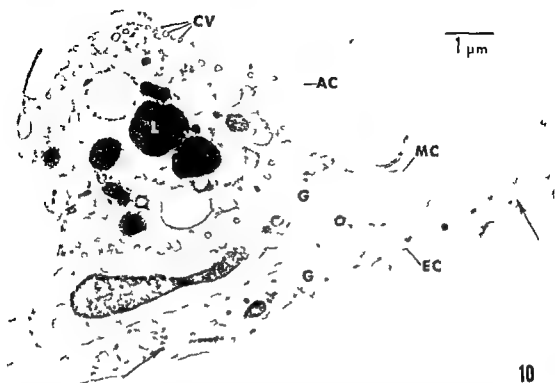
lar clefts (IC) are variously dilated between the apical region of the junctional complex (lower center) and the basal more constricted openings on the basal lamina (BL). The invaginations of the basal surfaces of the epithelial cells (BD). Coated vesicles (arrows) containing 6-hydroxydopamine.

6-hydroxydopamine

cogen at all ages, and polysaccharide particles were still present in this region at 72 days. Moreover, the mitochondria became of giant size in the older animals with some of them

measuring up to 1 μ m in diameter compared with 0.2 to 0.4 μ m at 11 days.

There was an increased tendency for spherical structures resembling macrophages



10

10 The mesothelial layer of Reissner's membrane in aged rats often showed adjunct cells (AC) as shown. Such cells contained numerous lysosomes (L) and were highly vesicular with both coated (CV) and uncoated (UV) present. In this particular sample of

the tissue the intercellular clefts (irregular spaces) of the epithelial cells (EC) are uniformly narrow. Glycogen is present in small patches (G). Golgi complex (M) mesothelial cell 17 day-old treated rat. 4 doses 6-hydroxydopamine

ly to become perched on top of closely related to the cells of the mesothelial layer (Fig. 10). These cells contained a usual complement of organelles but were chiefly characterized by their many lysosomes and numerous vesicles of various sizes. Many of the vesicles were coated. Uncoated vesicles were large and sometimes contained a flocculent material, lending a cystic appearance to the cells.

Epithelial cells

Three principal effects of the chemical sympathectomy on the epithelial cells of Reissner's membrane were: 1) glycogen accumulation occurred; 2) vesicles were numerically decreased; and 3) the intercellular clefts

narrowed and became of relatively uniform width.

Glycogen accumulation was the first obvious effect observed in the epithelial cell layer with the polysaccharide particles evident in quantity by 11 days (Fig. 4). The particles were electron dense and 150–300 Å in diameter. Polysaccharide particles increased numerically during further postnatal development, becoming accumulated into large aggregates in many of the epithelial cells by 17 days (Fig. 5). At both 17 and 32 days the aggregates were sometimes so large, particularly in the lateral cell regions, that they displaced the organelles ordinarily found in the area (Figs. 5–7). Glycogen particles were still present in aggregates in many of the epithelial cells at 72 days (Fig. 8), although other cells

a sharp decline in the number of these particles

Within the aggregates, the glycogen particles often had a tendency to group in linear arrangements or strings (Fig 8). Clusters of particles partially enclosed by what appeared to be collapsed membranous material were occasionally present.

Coated vesicles were of typical numbers in the epithelial cells of the membrane in the 11 day old treated rats and the intercellular clefts were generally tortuous and dilated. By 17 days however, the number of coated vesicles was visibly reduced. The intercellular clefts varied in dimensions from one place to another (Fig 5). In some cases, the clefts were distinctly narrowed for a distance below the apical junctional complexes and above the basal outlets, with more typical dilatations present in between. In other instances the portions of the clefts visible in the micrograph were uniformly narrow resembling the condition commonly observed at 72 days of age (Figs 6 & 7). The apical junctional complexes and the basal openings retained their typical spatial relationships throughout. An appreciable increase in numbers of coated vesicles occurred in those epithelial cells showing decreased glycogen content at 72 days (Fig 9) but the clefts between all the epithelial cells of Reissner's membrane were more typically tortuous and variably dilated at this time.

The epithelial cells at the juncture region with the spiral limbus contained much glycogen at every age examined. At 72 days the particles were sometimes dispersed in the cytoplasmic matrix but in other instances large aggregates of glycogen were present.

DISCUSSION

Although numerous fluorescence and ultrastructural investigations of the inner ear have supported the original findings of Spoendlin & Lichtensteiger (1966) that there are perivascular and blood vessel independent groups of adrenergic fibers in the inner ear (Terayama

et al 1966 1977 Ross 1971 1973 Denzer 1974) the functional significance of this extensive sympathetic innervation has remained elusive. Stimulation or surgical extirpation of various portions of the cervical sympathetic chain where adrenergic fibers of the eighth nerve are said to originate (Spoendlin & Lichtensteiger 1967, Terayama et al 1968) leads to changes in cochlear microphonic activity (Seymour & Tappin 1953 Beckert et al 1956), but the precise relationship of the observed changes to manipulation of one or the other of the two groups of adrenergic fibers has not been demonstrated conclusively. Neither does interruption of the cervical sympathetic chain at the superior cervical ganglion result in more than equivocal changes in inner ear morphology after survival periods of up to 22 days (C G Wright personal communication).

Chemical sympathectomy with 6-hydroxydopamine is a powerful tool to use in experimental studies of long term possibly homeostatic functions of the adrenergic nervous system in the inner ear. The drug can under appropriate conditions of critical dosage (Thoenen & Tranzer 1968 Jonsson & Sachs 1971, Tranzer 1971) cause reversible selective destruction of peripheral adrenergic nerve terminals in the adult (Tranzer & Thoenen 1968 1968 Malmfors & Sachs 1968) and irreparable damage to postganglionic cell bodies in the newborn (Angeletti & Levi Montalcini 1970).

The technique is not without its pitfalls however. An important time factor may exist (Saner & Thoenen 1971) and species differences are marked (Malmfors 1971). Moreover extensive destruction of the sympathetic system is essential if specific effects of catecholamine deprivation are to be studied for even slight sparing of adrenergic terminals may be sufficient to maintain baseline activity of some target organs once supersensitization to circulating catecholamine develops (see Lundberg 1971). The situation is complicated further in adult animals in which repair is rapid with complete anatomical regeneration

adrenergic nerve terminals possible within months (Hacuser et al., 1969, Jonsson & Hs, 1970, de Champlain, 1971) although functional return occurs much earlier (Jonsson & Sachs, 1972). The experimental procedure of repeated injections of 6-hydroxydopamine utilized in this study was planned taking into account the disadvantages noted above and was based upon methodology developed earlier by Thoenen (1971).

Chemical sympathectomy followed by ultrastructural study of inner ear morphology has been carried out previously in the newborn (Ross, 1972, Ross et al., 1974) and in the adult rabbit (Densert, 1975). In these instances, evidence of damage to the organ of Corti was obtained. The current series of experimental rats also showed structural alterations in the organ of Corti, particularly in the region of the inner hair cells, which are under investigation. Such evidence of deletions in morphological change is not conclusive because it is subject to question on the basis of possible artifact, or on grounds of some degree of cell specific toxicity, as noted by Densert (1975).

The positive finding of extensive glycogen accumulation in Reissner's membrane following chemical sympathectomy cannot be attributed to artifact nor to a toxic action of the procedure on the affected cells. Such storage requires synthetic activity and viable cells. Moreover, study of animals sacrificed at various times clearly shows first a progression, then a regression, of glycogen accumulation and related events (vesicle reduction and narrowing of the intercellular clefts of the epithelial cells) between 11 and 72 days. Partial return of conditions to a more typical state some time during the longest survival period may coincide with regeneration of at least some of the peripheral adrenergic fibers. This would suggest that, even under our experimental method of repeated injection of new animals with 6-hydroxydopamine, systemic destruction of peripheral adrenergic neurons was not complete. The overt signs of

autonomic imbalance noted do indicate, however, that neuronal damage was extensive.

Glycogenolysis in liver and some other cells is under the regulation of the adrenergic nervous system through the intracellular, second messenger cAMP, as described by Sutherland and his colleagues (Sutherland, 1950, 1951, Sutherland & Rall, 1960, Robison et al., 1967, 1971, Sutherland et al., 1968, and many others). When circulating catecholamine levels are high, such cells are stimulated at specific β -adrenergic receptor sites (Robison et al., 1967) on their cell membranes, where binding of the amine occurs, to activate adenylate cyclase to catalyze conversion of ATP to cAMP. This starts an "amplification cascade" which results in a breakdown of glycogen to glucose and in utilization of all glucose precursors to form free glucose. Moreover, not only is glycogen catabolism stimulated, but glycogen synthesis is prevented. Conversely, in the absence of appropriate levels of catecholamine, dissociation of the bound neurohumor occurs. This suppresses the cascading effects which lead to glycogen utilization but sets in motion enzyme systems promoting glycogen storage.

It would seem apparent from the present results that there must be similar β -adrenergic receptors on the cell membranes of the epithelial cells of Reissner's membrane which ordinarily function (in the presence of sufficiently high levels of catecholamine) to set in motion the appropriate enzyme systems to insure the availability of glucose and to prevent glycogen formation. Under conditions of catecholamine deprivation, as in the present case of chemical sympathectomy, glycogen accumulation was favored. By size (~ 150 – 300 Å) and by arrangement as single units, the glycogen was in the form of β particles.

Because glycogen accumulation was not anticipated, even though catecholamines function in glycogen utilization and storage elsewhere in the body, no special techniques were used to preserve or to stain the polysaccharide particles. Procedures ordinarily employed by

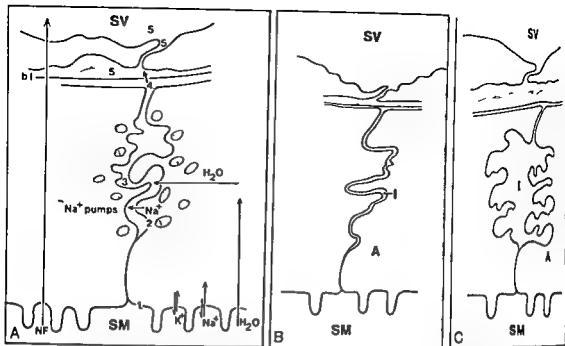


Fig 11A Figure 11A shows diagrammatically the sequence of events that could lead to net flow of $\text{Na}^+/\text{H}_2\text{O}$ across the epithelial cells of Reissner's membrane from scala media (SM) into scala vestibuli (SV). This sequence is as follows:

- Ions and water pass across the semipermeable membrane at the endolymphatic surface (1) into the cell passively, by diffusion.
- Active pumping of Na^+ occurs across the lateral cell membranes (2) into the intercellular clefts (3). This causes an ionic gradient for Na^+ at the endolymphatic surface of the cell (1) and an osmotic gradient for H_2O across the cell and into the cleft (H_2O arrows at right and center).
- The movement of the H_2O into the cleft increases the hydrostatic pressure there forcing fluid across the basal opening (4) and the basal lamina (bl).

- The narrow basal opening and the basal lamina constitute a barrier to free flow of fluid impeding but selectively restricting ion/fluid flow into phatic space (S) scala vestibuli (SV).
- The net flow of solute/solvent is shown at left (4 arrow).

Fig 11B C The degree of distension of the intercellular cleft is indicative of the functional state of the epithelial cells. In (B) the cells are relatively inactive and are moving solute/solvent in quantities sufficient to cause a gradient for water across the cell (A) and into the intercellular cleft (1). In (C) the cells are very active causing increased distension of the intercellular cleft (1) for the apical and basal ends of the cleft keep the relationships in both cases. SV: scala media, S: scala vestibuli.

us for transmission electron microscopy, however, routinely include primary fixation with a glutaraldehyde mixture and post-fixation in osmium, which closely approximates the recommendations of Revel et al (1960) for preservation of glycogen for ultrastructural study.

Why the epithelial cells mobilize glucose cannot be answered on the basis of the anatomical results alone, but narrowing of the intercellular clefts following glycogen accumulation in the treated animals is strong presumptive evidence that at least a portion of the glucose furnishes energy for a coupled solute-

fluid transport system across the cells. The concomitant decrease in the number of coated vesicles might or might not be related to the apparent reduction in such transport taking place across Reissner's membrane after chemical sympathectomy.

By analogy to other cell systems that concentrate solutes in enclosed fluids, such as the gall bladder (Kaye et al, 1966), active transport of ions, such as Na^+ , or other solutes into the intercellular clefts must normally create gradients both for the solutes and for water across the epithelial cells of Reissner's

membrane from the endolymphatic surface (Fig. 11). The diffusion of water from the endolymph across the cells and into the clefts would osmotically follow active pumping of solute into the intercellular spaces would increase hydrostatic pressure in the clefts. This would, in turn, force the solutes and solvent across the narrow basal openings of the clefts across the basal lamina into the perilymphatic compartment. The width of the intercellular clefts, then, should reflect the level of activity of the adjacent cells at any given moment. The extreme narrowing of the clefts that was seen by 32 days after initial treatment with 6-hydroxydopamine would indicate that active transport of solute into the spaces had totally ceased by that time.

If the final passage of the water and solutes from the intercellular clefts into the perilymphatic space depends upon differences in hydrostatic pressure and not upon active, selective transport, the fluids in the two compartments should normally be similar. This would suggest that the epithelial cell layer of Reissner's membrane, like many other epithelial tissues involved in fluid transport in vertebrates, may have a coupled, energy dependent, Na^+/H^+ transport system. Regardless of the solute transported, however, the coupled movement of water suggests that Reissner's membrane may play an important role in helping to maintain ion and fluid balance in the inner ear, a function that in turn would appear to be subject to catecholamine regulation. That the portion of the membrane attached to the limbus may be specialized for regeneration of damaged cells or for growth during early development has been suggested elsewhere (Ishiyama et al. 1970, Johnsson 1971).

In light of the present results suggesting a coupled solute and fluid transport capability of the epithelial cells, it is interesting that chemical analytical studies indicate that transport $\text{Na}^+-\text{K}^+-\text{ATPase}$ activity in Reissner's membrane is much lower than that of the stria vascularis (Kuppers & Bonting, 1968, Matschinsky & Thalmann, 1970). Matschinsky

and Thalmann have pointed out that the amount of activity in the membrane is nevertheless as great as that found in many other secretory organs that pump cations. It should be noted that these studies were carried out on the two-layered membrane, whereas our morphological findings would indicate that the active solute transport is confined to the epithelial layer of Reissner's membrane.

With respect to the finding of extensive glycogen accumulation in the epithelial cells of Reissner's membrane following chemical sympathectomy, it is surprising that chemical analysis has shown the activities of the enzymes involved in glycolysis (Thalmann et al., 1970) and glycogenolysis (Matschinsky, 1970) to be relatively low (See also Thalmann, 1971). This finding must be reconciled with another, however, which demonstrated that phosphorylase a increased nine fold in Reissner's membrane within three minutes under the stress of ischemia, but only about three fold in the stria and one and one half times in the organ of Corti (Matschinsky & Thalmann, 1970). Phosphorylase a is the active form of the enzyme and is involved in glycogenolysis. Unfortunately, histochemical studies of adenylate cyclase localization in the membranous labyrinth, which could shed some light on possible sites of catecholamine regulation on the epithelial cells, have yielded only equivocal results (Kerr & Schacht 1976, and personal communication) while recent biochemical analytical findings have indicated that the level of adenylate cyclase activity in Reissner's membrane is extremely low (Palaheimo & Thalmann, 1977). However, recent quantitative studies by Thalmann and Thalmann (1978) indicate a substantial activity of adenylate cyclase in Reissner's membrane (about 7.5 picomoles/mg dry weight/min).

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ZUSAMMENFASSUNG

Ratten wurden durch mehrfache Injektionen von 6-Hydroxydopamin während der frühen postnatalen Entwicklung chemisch sympathektomisiert. Kontrollen vom gleichen Wurf blieben unbehandelt oder wurden nur mit Lösungsmittel injiziert. Innenohrgewebe von einer Serie behandelter und unbehandelter Ratten im Alter von 11

Membran, vor allem in den Epithelzellen nahe dem Limbus spiralis. Die Epithelzellen in diesem Gebiet besaßen auch raues endoplasmatisches Retikulum mit stark erweiterten Zisternen, die eine granulare Substanz enthielten. Später als 11 Tage war kein Glykogen vorhanden, und die Zisternen verkleinerten sich mit weiterer postnataler Entwicklung. Die Epithelzellen der Membran in Kontrolltieren aller Altersstufen hatten interzelluläre Spalten, die an der endolymphatischen Oberfläche von Zonulae occludentes verschlossen waren, am basalen Ende eine enge Öffnung hatten und dazwischen verschiedene weit waren. „Coated vesicles“ wurden häufig an apikalen und lateralen Zellgrenzen sowie intrazellulär gefunden. Behandelte Ratten hingegen zeigten eine Anreicherung von Glykogenpartikeln in den Epitheln, die zwischen dem 11 und 32 Tag anstieg und in einigen Zellen 72 Tage vorhielt. Die Glykogenpartikel waren vom β -Typ, elektronendicht, ~ 150 - 300 Å im Durchmesser und waren oft zu großen Massen aggregiert. Vor allem in den lateralen Zellregionen. Gleichzeitig mit der Glykogenanreicherung nahm die Zahl der „coated vesicles“ ab und die interzellulären Spalten verengten sich, bis sie nach 32 Tagen gleichmäßig eng waren. Eine Zunahme der „coated vesicles“ und eine Erweiterung der interzellulären Spalten wurde nach 72 Tagen beobachtet. Die Ergebnisse werden dahingehend interpretiert, daß die Epithelzellen der Reißnerschen Membran die Fähigkeit haben zu einem energieabhängigen gekoppelten Ionen/Wasser-Transport, der normalerweise vom Glukosemetabolismus abhängig ist. Es scheint, daß der Glukosemetabolismus von Katecholaminen reguliert wird, was wiederum auf die Anwesenheit von β adrenergen Rezeptoren auf den Epithelzellen der Reißnerschen Membran schließen läßt.

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MECHANICAL PROPERTIES OF BASILAR MEMBRANE

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Abstract A fresh basilar membrane has different mechanical properties in the radial and in the longitudinal directions. When pressure with a needle is exerted on the basilar membrane a narrow radially oriented strip is deformed. The form of the deflection can be deduced from pathological consequences of the acoustic trauma as well. The observed anisotropy is a property of the vital membrane and is disturbed by chemical and physical injuries and is lost post mortem. The post mortem changes can explain the results obtained by von Békésy and differ from ours. The physiological meaning of the mechanical properties of the basilar membrane is discussed here.

The theories and hypotheses about cochlear mechanics as well as the physical and mathematical models of the cochlea generally assume that the cochlear partition is a homogeneous substance, dynamical properties of which are similar to those of the rubber membrane. However, the mode of the partition vibrations on acoustic stimulation is not dependent only on the hydromechanical forces transmitted to the fluids of the inner ear by the tapes but also on the sum of mechanical properties of the individual structures of this morphologically very complicated organ. According to the histological findings obtained up to date with respect to mechanics, cochlear partition can be considered to be an inhomogeneous body composed of components of differing rigidity, elasticity and plasticity.

This paper deals with the mechanical properties of the basilar membrane. The basilar membrane (BM) consists of the basic amorphous substance and radially oriented fibres. In

pars tecta these fibres are arranged densely, one along the other, in pars pectinata the single layer is divided into two. The upper layer which is near to the vestibular surface of the membrane is thin and compact, under it there is a layer of fibres grouped into bundles visible even under a light microscope. Helmholtz took them for resonant strings.

MATERIAL AND METHODS

Six week old guinea pigs weighing between 300-330 g were decapitated, their temporal bones removed and bullae opened. After the removal of the bony cochlear shell, individual turns were exposed to gain access to the cochlear partition. Under a stereomicroscope using dethermal filter lighting cells of the Corti organ were removed from the basilar membrane with a fine cotton wool brush. During the dissection the tissues were wetted with the Ringer solution. Above the decellularized BM a thin glass or metal needle with a rounded point of the diameter not exceeding 40 μm was fixed in Zeiss-Jena micromanipulator. During the measurement the needle was moved perpendicular to the surface of the BM. At a suitable angle of the incident dethermal filter light, the membrane and the changes of its surfaces were distinctly visible. BM can be slightly stained with toluidine blue dye added to the Ringer solution, for the purpose of photo documentation, BM has to be stained more strongly.



Fig 1 Microphotograph of the fresh basilar membrane deflected by the needle. The deflection has a shape of a narrow radially oriented strip. Inset: Key N needle, BM basilar membrane, L spiral limbus

Besides the fresh cochleae, i.e. those examined within 15 minutes of decapitation, the same method was used to examine the mechanical properties of BM in cochleae obtained from animals only after 24 hours after decapitation. During the elapsed time the temporal bones were kept at a temperature of

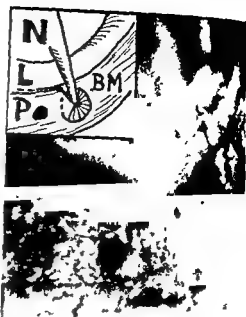


Fig 3 Microphotograph of the basilar membrane

N needle, L spiral limbus P partition
BM basilar membrane

5–10°C. Temporal bones of other animals were fixed in a 5% and 10% formaldehyde solutions, in formaldehyde solution with soap addition in a 5% and 10% lysol solutions. The reason for these examinations will be dealt with in the discussion.

RESULTS

By gradual pressure of the needle on the vestibular surface of the BM, there appeared in all turns of fresh cochleae the same form of membrane deformation. A narrow, radially oriented strip was deflected. Its length occupied the entire radial size of the BM, i.e. from the spiral ligament up to lamina spiralis ossea, but its width was determined by the needle size. The thinner the needle the narrower was the deflected strip of the membrane. The deformation did not spread along the partition in the longitudinal or spiral direction (see Fig 1). Under the needle a groove appeared in the form of parallel strings deflected by the pressure of a narrow object (see Fig 2).

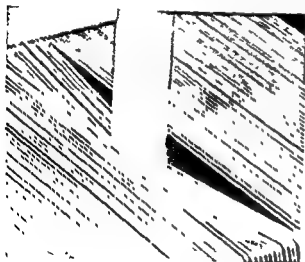


Fig 2 The deflection of the BM



Fig. 4. Microphotograph of basilar membrane after the removal of Corti's organ cells. After the displacement of 1 μ m the basilar membrane has its normal appearance of the radial fibrous system.

When the needle was removed, the BM assumed its original position. When a needle ressure was applied to cochleae that were not fresh, but dissected up to 24 hours after the death of the animal, a circular impression appeared, similar to that which appears when pressure is exerted on an elastic homogeneous membrane or on the skin on a soft tissue layer. A similar circular deformation was found on the deflected basilar membrane in cochleae fixed with formaldehyde, with formaldehyde with soap addition, and on specimens deposited in lysol solution. The impression in all the cases was always rounded, like a broad shallow crater. If the needle shift was too big and exceeded the BM elasticity stress limit so that the BM broke, the perforation of the fixed BM was always circular (Fig. 3). However, the orated hole in fresh membranes closed immediately after the removal of the needle so that the rupture became invisible even when

the membrane was examined at high magnification in a light microscope (Fig. 4).

In specimens fixed with a 5% and 10% lysol solutions, the epithelium of the inner ear was considerably damaged, the basilar membrane was similarly deformed by a circular impression when pressure was exerted with a needle, yet the membrane was not so solid and it tore easily.

DISCUSSION

In 1941, Georg von Békésy published a paper in German on the elasticity of the cochlear partition. The paper also described the form of the BM deformation caused by the pressure of a thin glass needle or a human hair. In the apical turns of the human cochlea he found a circular depression, in the basal part of the cochlea near the stapes the deformation was ellipse shaped with the longitudinal ellipse axis corresponding to the longitudinal direction of the cochlea. From his experiments, von Békésy deduced that the BM had equal mechanical properties both in the radial and in the longitudinal direction and that the BM behaved as a homogeneous membrane without any mechanical orientation. He supposed that the eventual strengthening by the radial fibres is compensated for by the longitudinal elements of the tympanic layer (*tympanale Belegschicht*) which "in der Längsrichtung der Membran verlaufende Fasern zeigt". Hence the idea originated that the basilar membrane has no mechanical orientation and when deflected it behaves as a homogeneous membrane, e.g. a rubber membrane.

It is evident from both the German original of the paper and its English translation (1948) that von Békésy used human material for his experiments. In his book (1960), page 20, he writes "Fresh temporal bones are to be obtained from the prosector of a large hospital." It is evident that this material could not be fresh in the supravital sense, but it must have been several hours old, obtained most probably only the next day after death. On

those basilar membranes that were examined up to 24 hours after death, we have also observed circular impressions under the needle. The post-mortem changes after 24 hours damage the tissues of the Corti organ considerably, as described by Wersall et al. in 1965. Such changes might be overlooked in a low magnification stereomicroscope, especially if the tissue had not been stained and the tympanic wall had been observed.

The post-mortem changes increased during dissection when the specimens were immersed in tap water, as described by von Bekésy.

Further, page 433 of the English translation (1960) reads "Measurements made over a period of two weeks showed no important changes for a temporal bone kept in Lysol solution at the temperature of 5°C". The English translation is mistaken in the word *Lysol*. The German original states that the bones were kept "in Lysoformlösung". Lysoform was a trade mark name of a solution then widely used in Europe for external disinfection as well as for conservation purposes in anatomy. It did not contain any Lysol. Its formula reads: Formalin 40% 100 ml, Spiritus saponis kalini 50 ml et aquae dest. ad 1000 ml. That is why we have used in our experiments not only the formaldehyde solution but also one with soap addition conforming to the Lysoform formula. Under the influence of the English version we have also examined the effect of the Lysol solution at 5% and 10% concentrations. Von Bekésy's original papers never mentioned Lysol. It can be seen that Lysol damages the tissues to a great extent, so that they soon become almost useless for experimental purposes. However, even the formaldehyde solution fixation changes the chemical and physical properties of the tissues significantly, so that their mechanical properties become markedly different from those of the *in vivo* condition. This was shown on fixed specimens. When the BM is deflected by the needle it becomes considerably more rigid and the deformation appears as a relatively broad circular crater. Furthermore, we have

observed that the fixed membranes tear at smaller deflections than do the fresh ones. It is not necessary to deal in detail with the effect of the fixation on individual tissue and cellular components. It is a matter of common experience that the mechanical properties of biological objects change after having been fixed in formaldehyde.

The experiments with fresh BMs unambiguously show their mechanical orientation. The membrane can be presumed to deflect in narrow segments also when stimulated by noise. This is confirmed also by our previous observations when the histological consequences of acoustic trauma were analysed. After exposure to high intensity sounds, tiny perforations were found in the BM. They were usually elongated, radially orientated and always of a very small diameter. The BM perforations never joined, even when closely adjacent to one another. These findings caused by intense noise certainly corroborate the experimental results obtained with needles.

The high sensitivity of the hearing organ from the aspect of cochlear mechanics can be better explained by anisotropy of the BM. For a deflection of the BM in a narrow radial step a smaller volume of a liquid is necessary than for a greater deflection of a circular shape. This is important for the auditory threshold. A narrow deflection also facilitates better peripheral analysis of frequencies. Although the original Helmholtz theory of the resonance of the BM strings tuned to the individual frequencies cannot be accepted, it seems that the mechanical orientation of the membrane facilitates oscillation within very narrow and separated segments, similarly to hammers striking the dulcimer's strings. Of course, the cochlear membrane fibrils are not resonant strings but a limiting element which prevents the propagation of the deflection in the longitudinal direction in the basic plastic BM material of the cochlear partition. The converse is also true—the plasticity of the basic material facilitates the oscillation of the membrane in narrow segments lying parallel with the radial

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ZUSAMMENFASSUNG

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REDUCTION OF THE ENDOCOCLEAR POTENTIAL BY THE NEW "LOOP" DIURETIC, BUMETANIDE

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Abstract The effect of bumetanide upon the endocochlear potential (EP) was examined in 46 guinea pigs. The EP was reduced with dosages of 3 mg/kg or more. The maximum depression of the EP (reduction to -30 to -40 mV) was obtained at a dosage of 30 mg/kg. The recovery of the potential was incomplete at any dosage within three hours and the response pattern of the EP to bumetanide was similar to that to ethacrynic acid. The present experiments revealed that bumetanide, by weight, has a stronger ototoxic potency than the other loop diuretics—furosemide and ethacrynic acid. However, the diuretic effect of 1 mg bumetanide is equivalent to 40 to 60 mg furosemide or ethacrynic acid. Therefore the relative ototoxic potency of bumetanide is much smaller suggesting that from a clinical standpoint bumetanide is much safer than the other loop diuretics.

Bumetanide (3 *n*-butylamino-4-phenoxy-5-sulfamoylbenzoic acid) (BUM) is a new "loop" diuretic whose primary diuretic action is exerted in the ascending limb of the loop of Henle, similar to the other "loop" diuretics ethacrynic acid (EA) and furosemide (FU) (Feit, 1971, Østergaard et al, 1972). EA and FU are known to have ototoxic side effects and have been shown to have a profound influence upon the endocochlear potential (EP) (Prazma et al, 1972, Bosher et al, 1973, Kusakari et al, 1977). Their main site of action in the cochlea is considered to be the stria vascularis (Quick & Duvall, 1970, Bosher et al, 1973). We could find only one report in the literature suggesting possible ototoxicity in man—a transient hearing loss following the administration of 1 mg BUM (Asano et al, 1974). Bourke (1976) reported three cases in which deafness occurred following the administration of FU, but the clinically equivalent diuret-

ic dosage of BUM (one fortieth that of FU by weight) showed no evidence of ototoxicity. Brown (1976) reported that BUM exerts pronounced effects upon the EP in the cat. Since the guinea pig is widely used in experimental studies, and since "loop" diuretics show wide variations in diuretic effects among various species, it seemed relevant to determine the action of BUM upon the EP in this animal.

MATERIAL AND METHODS

Forty-six albino guinea pigs weighing 250 to 350 g with normal Preyer pinna reflex were used. Tracheostomy was done under pentobarbital anesthesia (28 mg/kg) and the animal was artificially respired after intraperitoneal injection of succinylcholine chloride. The left bulla tympanica was opened wide via a ventrolateral approach. The electrode (a glass micropipette with a tip diameter of about 2 μ m, filled with 150 mM KCl, and connected to a Grass P14 d microelectrode preamplifier by means of a chlorided silver wire) was advanced via the round window, through the basilar membrane and into scala media. The EP of $+80$ to $+90$ mV (with reference to the potential in scala tympani) was recorded with a pen oscillograph; the indifferent electrode was placed on the exposed neck muscles. BUM solution in dosages from 2.5 to 40 mg/kg was administered over a period of 3 minutes through an indwelling catheter in the right jugular vein. The EKG was monitored throughout the ex-

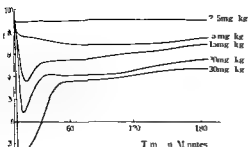


Fig 1 The effect of intravenously applied bumetanide on the endocochlear potential in the guinea pig. The drug was administered at the indicated dosages over a period of 3 min via an indwelling catheter in the right carotid vein

ment and the body temperature was maintained at about 37°C

RESULTS

The initial magnitude of the EP in the present experiment was 84.8 ± 4.8 mV (means \pm S.D., $n=46$). No effect upon the EP was seen in 2 animals given 2.5 mg/kg of BUM, however, at 5 mg/kg and above, a dose dependent reduction of the EP was obtained. Typical response patterns are shown in Fig 1. After a latent period of one to two minutes, the EP began to decline rapidly, reaching its lowest level within 6 to 15 min (except in the case of 5 mg/kg, at which the EP declined on a much slower time scale). The maximally reduced levels were 72.0 ± 4.4 mV ($n=3$), 54.0 ± 13.1 mV

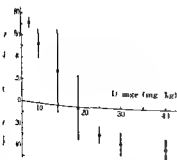


Fig 2 The effect of intravenously applied bumetanide upon the endocochlear potential expressed as a function of drug dose. Data points represent means \pm S.D.

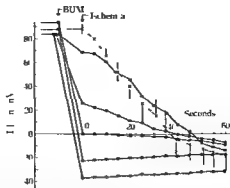


Fig 3 Effect of ischemia upon the EP of bumetanide intoxicated ears (●—●). The dotted curve indicates the decline rate of the EP in untreated control ears (mean \pm S.D., $N=21$). Adapted from Kusakari et al. 1977.

($N=3$), 30.7 ± 37.4 mV ($n=12$), -0.8 ± 29.4 mV ($n=11$), -25.0 ± 7.5 mV ($n=4$), -31.9 ± 10.6 mV ($n=7$), and -35.8 ± 9.4 mV ($n=4$) for dosages of 5, 10, 15, 20, 25, 30, and 40 mg/kg, respectively (Fig 2). After the maximally reduced level was reached, a biphasic recovery set in (i.e. an initial rapid recovery period, followed by a slow progressive one), however, in no case was complete recovery seen within the maximum observation period of three hours, with the higher dosages (30 and 40 mg/kg) the EP recovered only to +30 to +40 mV during this time. Although a great deal of variation in response between animals was seen at dosages of 15 and 20 mg/kg, the responses were quite reproducible with the higher and lower dosages.

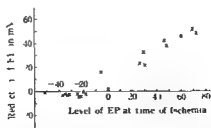


Fig 4 The effect of bumetanide intoxication upon the ischemic decline of the endocochlear potential. Ischemia was induced by sectioning the aorta when the endocochlear potential had reached its maximally reduced level in response to bumetanide (abscissa). The ordinate represents the reduction of the potential from the preischemic level after 35 sec of ischemia.

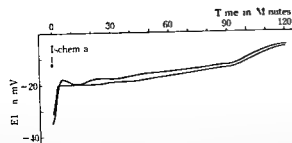


Fig 5 The effect of ischemia upon the endocochlear potential of ears severely intoxicated by bumetanide illustrating the reduction of negativity which occurs when the level of the endocochlear potential is reduced to below -15 to -20 mV in response to the drug

In a second series of 20 animals, the aorta was sectioned when the EP had reached its maximally reduced level in response to various dosages of BUM. As shown in Figs 3 and 4, the ischemic decline rate of the EP in these pre-intoxicated animals decreased with the degree of primary damage induced by the drug, however, when the maximally reduced level was lower than about -20 mV, ischemia caused a decrease in negativity. In the 2 cases shown in Fig 5, the EP began to rise within 10 sec after the onset of ischemia, and increased from a level of primary damage of about -30 and -35 mV, respectively, to about -20 mV within 10 minutes. After remaining at this level for 30 to 40 minutes, the EP gradually increased toward the baseline. A reduction of negativity was also seen in superimposed respiratory hypoxia. The EP returned to the preasphyctic level upon reventilation (Fig 6).

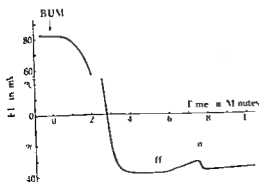


Fig 6 The effect of respiratory hypoxia (off) and subsequent reventilation (on) upon the endocochlear potential of ears severely intoxicated with bumetanide

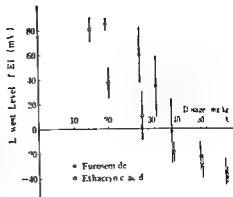


Fig 7 The effect of intravenously applied ethacrynic acid (EA) and furosemide (FU) upon the endocochlear potential of ears severely intoxicated with bumetanide

1977)

DISCUSSION

EA, FU, and BUM are potent diuretics which act primarily in the ascending limb of the loop of Henle. Although there are minor differences, these drugs share a common mechanism of action in the nephron (Davies et al, 1974; Olesen et al, 1973; Kim et al, 1977; Stein et al, 1968; Østergaard et al, 1977). There are many clinical reports on the ototoxicity of EA (Schneider & Becker, 1966; Pflüger et al, 1969), and FU (Schwarz et al, 1977). Electrophysiological, biochemical, and morphological studies on the inner ear suggest that their primary site of action is the stria vascularis and that the EP is the primary electrophysiological parameter involved (Prazma et al, 1977; Kusakari et al, 1977; Quick & Duvall, 1970; Kusakari et al, 1977). Although both drugs reduce the EP, the response pattern is quite different—namely, the recovery of the EP is complete within 2 hours after administration of EA, whereas the EP returns to its original level within one or two hours even with high dosages of FU (Kusakari et al, 1977). Brundage (1976) studied the effects of BUM upon cochlear potentials in the cat and showed that the duration of ototoxic action of BUM is similar to that of FU and less than that of EA. However, the results obtained in the present study in the guinea pig indicate that the response pattern of the EP to BUM more closely

mbles that of EA. Species differences have not been recognized in the case of EA and FU.

Fig 7 shows the maximally reduced level of the EP induced by various dosages of EA and FU in the guinea pig (Kusakari et al 1977). In a comparison of the three diuretics shown in Figs 2 and 7 it is evident that on a weight basis BUM is the most potent ototoxic agent, followed by EA and by FU. However, 1 mg of UM exerts a diuretic effect equivalent to 40–60 mg FU (Olesen et al 1973; Davies et al 1974) and EA is reported to have an absolute potency almost identical to or slightly greater than that of FU (Laragh, 1967). Therefore when the diuretic dose-response relations and clinical dosages of these three drugs are taken into consideration the relative ototoxic potency of BUM is far lower than that of the other two drugs. The present study indicates that although BUM is potentially ototoxic, the possibility of deafness occurring in patients receiving therapeutic dosages of the drug is remote because of its relatively smaller ototoxic potency per clinical dose.

Kuypers (1969) proposed that the normal EP consists of two components: a large positive electrogenic potential (100 to 120 mV), thought to be produced by active K^+ transport in the stria vasculans, and a small negative potential thought to be a K^+ diffusion potential (–20 to 40 mV). Although there is some disagreement concerning the exact nature of the two components (e.g. Honrubia et al 1976), a dual nature of the EP seems to be supported by most investigators.

Bosher et al (1973) proposed that the decline of the EP in the early stages of EA intoxication is due to the abolition of the positive secretion potential consistent with this proposition is the behavior of the high energy phosphates of the stria vasculans in EA intoxication which suggests a pronounced inhibition of energy utilization (Thalmann et al 1973; Kusakari et al 1977). However, there appear to be moderate effects upon energy generation as well.

Another line of evidence, originally thought to represent an additional reflection of a reduced metabolic rate, is the fact that the ischemic decline rate of the EP predamaged by EA is much lower than that in non-intoxicated animals. As shown in Figs 3 and 4, analogous results were obtained in the present studies with BUM, however, since ouabain exerts a much stronger inhibition of the ischemic decline of high energy phosphates but a distinctly lesser reduction of the ischemic decline rate of the EP as compared with EA, we no longer feel that this is a valid indicator of a reduced energy use rate (Kusakari et al 1977).

When the EP was reduced to a level lower than –15 to –20 mV by BUM superimposed ischemia produced an elevation of the potential. The presence of this anoxia-sensitive negative potential was also observed in the case of EA and FU (Thalmann et al 1973; Sellick & Johnstone 1974; Kusakari et al 1977). An analogous pattern in EA-intoxicated animals was reported in the utricular and the ampullar EP (Sellick & Johnstone 1974; Kusakari & Thalmann 1976). It is not clear at this point whether this is a normally existing potential unmasked by EA or FU administration or an abnormal potential which appears only in intoxication with loop diuretics.

Bosher et al (1973) suggested that the initial decline of the EP in EA intoxication is due to inhibition of stria enzymes, followed by a more prolonged phase of altered membrane permeability. On the basis of *in vivo* and *in vitro* experiments with ouabain which indicate remarkably similar effects upon the EP and upon stria Na^+K^+ ATPase, Kuypers (1969) postulated that this enzyme is involved in the generation of the EP. Since the effects of EA upon the EP are similar to those produced by ouabain, the assumption that the effects of EA are also due to interference with this enzyme did not seem unreasonable. However, recent studies (Kusakari et al 1977; Paloheimo & Thalmann 1976) demonstrate that the inhibition of stria Na^+K^+ -ATP

EA *in vitro* requires concentrations 500 times higher than those required to inhibit the EP by local administration. These results are in agreement with the report of Kuypers & Wilberts (1976) on the differential effects of EA and ouabain upon strial Na^+K^+ -ATPase activity.

By contrast, the inhibition curves of the EP and strial adenylate cyclase activity *in vitro* with respect to EA are very similar. Marked effects of EA upon adenylate cyclase and cyclic AMP, and a possible role of these two substances in ion transport and permeability have been described in other organs (Ede, 1974; Tria et al., 1974). Although the effects of EA upon strial adenylate cyclase are consistent with the proposition that interference with this enzyme may be the mechanism by which the EP is affected (Ahlstrom et al., 1975; Paloheimo & Thalmann, 1976), further studies are necessary to decide whether or not there is a causal relationship.

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ZUSAMMENFASSUNG

Der Effekt von Bumetanid auf das endocochleare Potential (EP) wurde bei 46 Meerschweinchen untersucht. Dosen von 5 mg/Kg oder darüber reduzierten das EP. Eine maximale Senkung des EP (Verminderung auf -30 bis -40 mV) wurde mit einer Dosis von 30 mg/Kg erzielt. Die Erholung des EP war bei allen Dosen unvollständig innerhalb der Beobachtungszeit von drei Stunden. Die Reaktion des EP auf Bumetanid war ähnlich wie diejenige auf Ethacrynsäure. Die Experimente lassen erkennen, daß Bumetanid (bezogen auf Gewicht) eine stärkere ototoxische Wirkung hat als die anderen loop Diuretika Furosemid und Ethacrynsäure. Jedoch der diuretische Effekt von 1 mg Bumetanid ist derselbe wie derjenige von 40 bis 60 mg Furosemid und Ethacrynsäure. Deshalb ist die relative ototoxische Wirkung von Bumetanid geringer, was bedeuten würde, daß vom klinischen Standpunkte aus gesehen Bumetanid viel harmloser ist als die anderen loop Diuretika.

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THE DEVELOPMENT OF HAIR CELLS IN THE EMBRYONIC CHICK'S BASILAR PAPILLA

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Abstract During the 7th to 21st (hatching) days, hair cells of the embryonic chick transform from an undifferentiated epithelium into cylindrically shaped tall hair cells (THCs), pitcher shaped short hair cells (SHCs), or intermediate hair cells that share structural characteristics of the first two. By the 11th day, hair cell types are unambiguous.

Hairs (stereocilia and a kinocilium) were first identified on the 7th day and resembled the adult pattern the 13th. The nucleus occupies relatively less volume as hair cells increase in size, becoming positioned centrally in THCs and basally in SHCs. Nucleoli, which are prominent throughout development, remain conspicuous even in newly hatched chicks. The cuticular cone begins to form the 11th day. By the 10th day, sensory nerve endings synapse on the bases of the HCs, which by the 11th day develop synaptic bars. Although efferent neurites were in evidence as early as the 11th day, synaptic contacts and their cisterns were identified by the 19th day but may form earlier. Supporting cells transform from cylindrically to flask shaped cells with constricted necks and may secrete at least a portion of the tectonal membrane.

In recent years, the adult avian basilar papilla, the counterpart of the mammalian organ of Corti, has been examined ultrastructurally in the pigeon (Cordier 1964a, b, Rosenhall, 1971, Takasaka & Smith, 1971), and cock (Janke et al., 1969). Within the basilar papilla of the auditory lagena, there are three types of hair cells: tall, short, and intermediate (Takasaka & Smith, 1971). These sensory cells are distinguishable on the basis of size, shape, stereociliary length, shape of cuticular plate or cone, and possibly innervation pattern. By comparison, the developing basilar papilla has largely been overlooked. In fact, the chick's cultured otocyst has served doubly as a model system for avian hair cell differentiation (Friedmann, 1959a, b, 1968, 1969) and also for the action of ototoxic antibiotics (Friedmann

& Bird, 1961, Friedmann 1965). In term cultured otocysts, however, hair cells apparently do not complete differentiation; only one of the three hair cell types is identifiable (Friedmann, 1969). The underlying factors responsible for the arrested differentiation are unknown. Recently, Hirokawa (1977) reported on aspects of hair cell development in normal embryos and those treated with β -4 gyrototoxin. In the present study, we follow the differentiation of hair cells in the embryonic chick's basilar papilla in order to compare the normally developing lagena to a and to cultured otocysts.²

MATERIALS AND METHODS

For this study, more than 250 White Leghorns were used. Groups of 1-4 dozen were incubated in a thermostatically controlled incubator that was set at $38 \pm 2^\circ\text{C}$ and were turned clockwise and counterclockwise at least twice a day during the incubation period. Prior to decapitation, the embryos were staged according to Hamburger and Hamilton's scheme (1951). Eggs from the 7th to (hatching) days were used primarily, a number within 4 days after hatching.

Immediately after the lagena tissue was exposed, it was placed in cold ($4-8^\circ\text{C}$) glutaraldehyde (3-3.5%) buffered in 0.1 M sodium

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² This represents an expansion of material presented at the Southeast Electron Microscopic Society, USA, 1977. (Cohen & Fermin 1977, Siegel, Fermin & Cohen 1977).

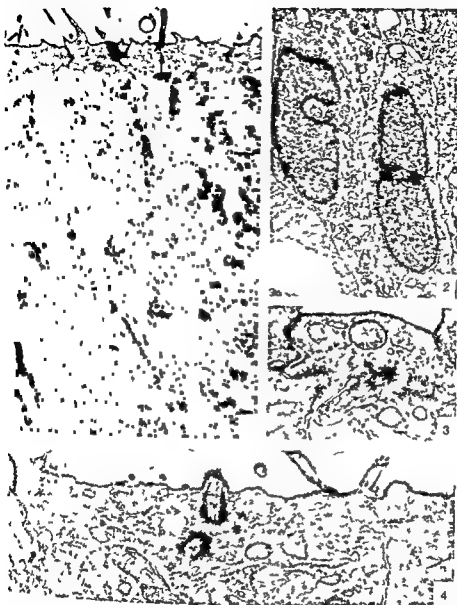


Fig. 1 Apical region of a cell. Microvilli and stereocilia are not distinguishable though a kinocilium (arrow) is present 7th day $\times 9000$
 Fig. 2 Invaginated nuclei at apical end of basilar papilla

possibly indicating high synthetic activities 7th day $\times 6000$

Fig. 3 End vacuole or ciliary body of Golgi complex 7th day $\times 29000$

Fig. 4 Diplosome in apex. Nascent kinocilium is emerging from surface. Microfilaments strengthen junctions 7th day $\times 29000$

acodylate and containing 5 mM Ca^{2+} , and less frequently in glutaraldehyde (2%) buffered in 1 M sodium phosphate, both at pH 7.2-7.4. After the lagena was freed from the temporal bone it was post fixed in buffered 1% osmium

tetroxide, pH 7.2-7.4, for 1-2 hours. The tissue was then dehydrated in either a graded series of acetone or reagent alcohol (50-100%), embedded in epoxy (Araldite 502) and sectioned with Sorvall's MT 1 ultramicro-

Table 1 Dimensional changes of hair cells (expressed in microns)

Hair cell type	Days of incubation									
	7th day		11th day		14th day		19th day		21st day	
	Undifferentiated epithelium		THC	SHC	THC	SHC	THC	SHC	THC	SHC
Hair cell length	9-13		12-14	7-8	10-16	8-12	15-23	9-13	17-24	10-14
Hair cell width	2.9-5.2		4-4.3	4.6-5.7	4-6	4.5-6.5	4-4.5	4-5	4.2-4.5	4-4.1
Nucleus long axis	2.5-6.1		4.7-6.5	4-4.5	5-8	4.3-5	5-8	4-5	3.8-7.7	6-7
Nucleus short axis	1.7-3.7		2.7-4.7	4-4.5	3-4.5	3.1-3.8	3-4.4	3-3.8	2.2-4.2	2.2-3.1
Cuticular cone	0		0.2-0.5		0.4-0.8		1.5-2.5		3.5-4.5	1-4.2
Tallest stereocilia	0.8-1.2		1.5-2.0		2.5-2.8		3-5.0		3-4.5	

some. The silver-gray sections were stained first with uranyl acetate (Watson, 1958) and then counterstained with lead citrate (Reynolds, 1963). Sections were examined and photographed with a Zeiss 9S-2 transmission electron microscope at magnifications ranging from 1900 to 21 000 and enlarged optically.

RESULTS

We used the anatomical terminology that was recommended by Takasaka & Smith (1971), which is more appropriate for the avian ear, as was the term 'cuticular cone' instead of 'cuticular plate' (Jørgensen, 1971). The central basilar papilla was used predominantly in the present study because of the presence of all three hair cell types.

7th day (Stage 31)

Because the pseudostratified epithelial cells of the basilar papilla appear uniform, presumptive hair and supporting cells can only be identified by relative positions, i.e., apical or basal, rather than by specific structural features or densities (Fig. 1). Cell divisions are infrequent but have not yet ceased as evidenced by mitosing cells, particularly the more apical cells. Many nuclei are deeply invaginated (Fig. 2), possibly indicating high synthetic activity. Centrioles of recently divided cells are centrally positioned prior to their apical ascent. A diplosome forming the kino-

cilium's basal body often displays as a vacuole or ciliary body of the Golgi complex (Fig. 3). A rudimentary kinocilium (Fig. 4) consisting of an axonemal skeleton that is usually arranged in a 8+2 pattern rather than the more common 9+2 of most other cilia, extends outward 0.7-1.2 µm from the apical surface; almost every cell. The other apical projections will form stereocilia or microvilli but a distinction between them is not presently possible (Fig. 1 and Table 1). Tight junctions and desmosomes zipper the apices of adjacent cells together. The tight junctions are strengthened by bundles of microfilaments (Fig. 4). Below these junctions, there are numerous extracellular spaces, which are largest at the basal regions of the basilar papilla and give a spongy appearance. There are also intracellular spaces or empty vacuoles (Fig. 1) that are sometimes limited by a membrane. Although resembling fixation artifacts, these empty vacuoles had once been filled with lipids that were extracted during tissue processing; they become less frequent subsequently. The basilar membrane forms on the buttressed bases of presumptive supporting cells by an underlying coating of amorphous extracellular material that is re-enforced with collagen fibers (Fig. 25). The nascent basilar membrane is not yet continuous with empty spaces through which many sensory neurites have already coursed. The neurites apparently do not wedge between crevices of adjoining cells. Although they have reached midway within the basilar papilla, the neur-



Fig. 5 First presumptive hair cell. It rests on superior fibrocartilaginous plate, close to columnar cells, and is identifiable by slightly denser cytoplasm. Microtubules traverse the cell's long axis. A forked stereocilium extends from the surface. In presumptive supporting cell, satellite

bodies encircle kinocilium's basal body. 8th day $\times 43000$.

Fig. 6 Midbody. The microtubules (approx. 230 Å in diameter), in this case 52, are remnants of the mitotic spindle. 8th day $\times 45000$.

have not yet established synaptic contacts with hair cells.

The cellular cytoplasm is quite dense and richly endowed with organelles that are evenly distributed. For example, polysomes become more numerous as differentiation proceeds, contributing later to the increased cytoplasmic density of hair cells. Chains of ribosomes, both free and attached to membranes, surround sacs of proteinaceous material. Later, once distinction between hair and supporting cells is possible, it becomes evident that these sacs are confined to supporting cells.

Compared with later stages of development, the mitochondrial population is small. In thin section, only 6–15 mitochondria are counted, each approximately 1.0–2.5 μm in length and

0.3–0.5 μm in diameter. Nestled within the mitochondrial cristae are opaque granules (500–700 Å) that are probably calcium deposits and remain as a consistent feature thereafter. The first traces of the tectorial membrane appear as a thin wisp of material hovering over the cellular projections (Fig. 1).

8th day (Stage 34)

Differentiation of hair cells begins first at the superior fibrocartilaginous plate where the presumptive tall hair cells are forming and then proceeds laterally across the basilar membrane towards the inferior fibrocartilaginous plate where presumptive short hair cells will form. In the former, there are several cells (14 μm in length) which are distinguishable

from the other by their slightly denser cytoplasm and are considered as nascent hair cells (Fig. 5). Occasionally rudimentary stereocilia sprout from their apices. However, their lack of other cytological landmarks prevents their unequivocal identification. Junctional complexes are also found in the superior sulcus, the transitional zone between the future columnar and hair cells.

In both hair and supporting cells, 10 to 15 granular satellite bodies (500 Å) encircle each kinocilium's basal body (Fig. 5). Mitochondria and microtubules are slightly polarized and aligned in parallel (paraxially) to the cell's long axis. Although the mitochondrial population has remained constant, the number of microtubules in the supranuclear region increased. A few secretory granules are dispersed within the cytoplasm (Fig. 5). The last few cells are completing division as evidenced by a midbody (Fig. 6), a remnant of the mitotic spindle.

9th day (Stage 35)

As hair cells become progressively denser, they now are more readily distinguishable from supporting cells, hair cell types, however, are not yet recognizable. Stereocilia grow outward from the hair cells and small microvilli sprout from the supporting cells. Although cytoplasmic organelles of hair cells begin to migrate, mainly to the supranuclear region, they do not congregate immediately beneath the stereocilia but instead circumscribe the area where formation of the cuticular cone will commence shortly.

10th day (Stage 36)

Afferent nerves establish the first synaptic contacts on the bases of hair cells. At the site of contact, the basal membrane becomes denser.

11th day (Stage 37)

Hair and supporting cells are now readily distinguishable from each other. Hair cells are shorter but denser, and in contrast, the sup-

porting cells are longer (about 2x) but less dense. Two hair cell types are now identifiable: 1) elongated tall hair cells (Fig. 7) narrow slightly into a neck whereas the stubby short hair cells (Fig. 8) display a truncated neck and flared collar. At their flat apical ends both tall and short hair cells buckle slightly into a slightly domed surface, perhaps a result of lateral expansion that will be particularly prominent in short hair cells.

The hair cells sprout stereocilia that increase in length in a stepwise fashion (0.25 µm), and, though not fully grown, are readily distinguishable from the shorter, narrower microvilli of the adjacent supporting cells (Figs. 7 and 8). The stem of each stereocilium is filled with a filamentous core that rows into a short dense neck through which a single rootlet extends downward to anchor into the nascent cuticular cone. The rootlet may indeed be the microtubules seen earlier the 8th day, and are now redeployed at 11th to associate with young stereocilia before the cuticular cone has started to form. The kinocilium is presumably nudged laterally by emerging stereocilia until it rests eccentrically within the cell but medially to the cell's axis and faces the superior fibrocartilaginous plate in the supporting cell, which lacks stereocilia. The kinocilium is centrally positioned.

In both tall and short hair cells as the cuticular cone begins to form it appears as a granular accretion that displaces organelles. It collects beneath the apical surface (Fig. 18). Multivesicular bodies appear but are confined to the supranuclear region as are secretory granules (0.4 µm in diameter).

A synaptic bar (Fig. 19) forms within the basal membrane at the site opposite the afferent terminal. At the site of established contact the previous dense basal membrane becomes less dense. Vesicles about 350 Å encircle the synaptic bars, and some also contact the plasma membrane. Tall hair cells are innervated by short efferent neurites, identified on the basis of cytoplasmic density and presence of vesicles, appear inside the basilar papilla but have not yet established synaptic contact (Fig. 22).

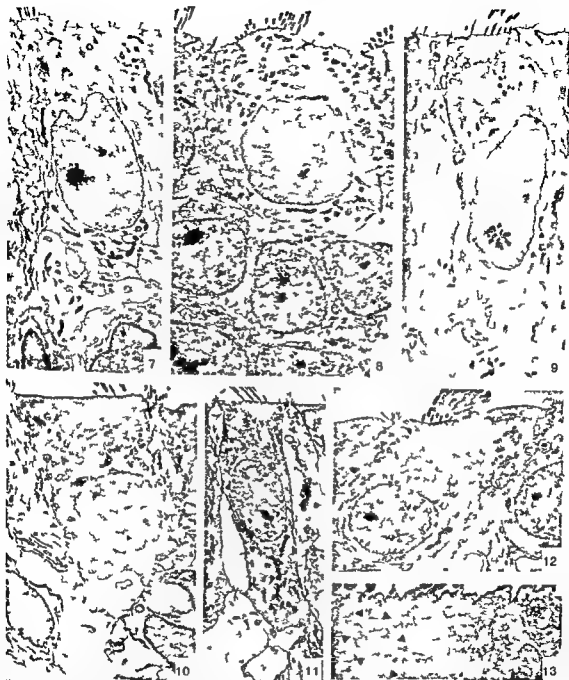


Fig 7 Naent tall hair cells lying on ledge of superior fibrocartilaginous plate (SFP). Because difference in position from the SFP to the inferior fibrocartilaginous plate these cells are darker than the former 11th day $\times 5000$

Fig 8 Short hair cells Stereocilia are shorter but more numerous than tall hair cells Apical surfaces are domed 11th day $\times 6000$

Fig 9 Tall hair cells Cylindrical shape is maintained as cell lengthen 14th day $\times 6000$

Fig 10 Short hair cell Spout is forming eliminating the previous symmetry 14th day $\times 6000$

Fig 11 Tall hair cells Cytoplasm is packed with free ribosomes organelles are polarized in both hair and supporting cells 20 days $\times 2500$

Fig 12 Short hair cell Pitcher shape resembles the adult 3 days after hatching $\times 5000$

Fig 13 Mitochondrion embedded within cuticular cone 4 days after hatching $\times 5000$

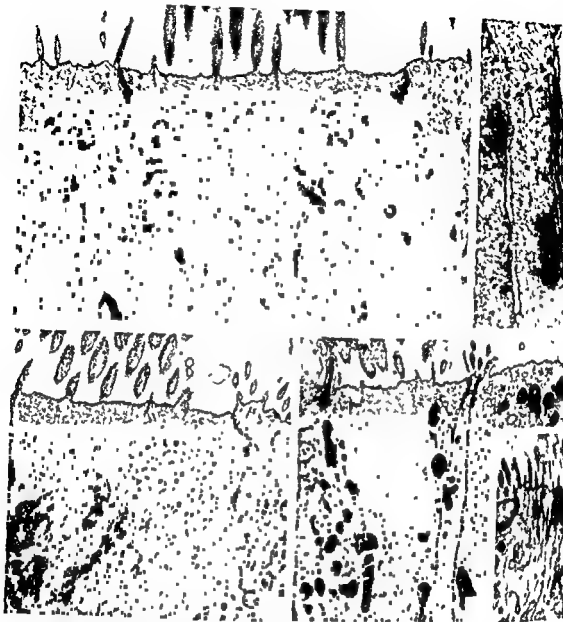


Fig. 14 Nascent cuticular cone of tall hair cell with full complement of synthetic organelles beneath it. Subsurface cistern runs paraxially (arrow). Supporting cells' cytoplasm is becoming progressively less dense, mitochondria are smaller than those of adjacent hair cell. 14th day $\times 19000$

Fig. 15 Surface cistern flanked by mitochondria. Subsurface cisterns, which are located apically or centrally, are identifiable earlier than the subsynaptic. 14th day $\times 35000$

Fig. 16 Cuticular cone in tall hair cell. Growth cones place by numerous

Fig. 17 Rootlets extend downward through cone. 3 days after hatching $\times 14000$

Fig. 18 Microtubules extending up into microvilli. 3 days after hatching $\times 19000$

The supporting cells are cylindrically shaped. Secretory granules, Golgi complexes, endoplasmic reticula (cisternal and tubular), and microtubules associated with the microvilli, become conspicuous. Mitochondria are

scarce, and in cross-section have small diameters than those in the adjacent hair cell. Supporting cells are permanently attached to the tectorial membrane by means of fibrous material that seems to extend from the

microvilli and also from the transient kinocilia. This thin fibrillar material arches over shorter stereocilia of the hair cells and forms a cupulla. Only the tips of the tallest stereocilia are embedded in the lower surface of the tectorial membrane, the shorter stereocilia, if attached at all, are embedded more shallowly and withdraw during fixation.

13th day (Stage 38)

Forming as early as the 7th day (Fig. 4), junctional complexes which are strengthened internally by bundles of cytoplasmic microfilaments but are not always apparent because of the plane of sectioning, join the apical ends of the hair and supporting cells to each other (Fig. 27). Immediately below the apical ends, forming (1–2 μm) tight junctions join hair and supporting cells together, and also adjacent supporting cells.

14th day (Stage 40)

The spout of the short hair cell, which will become pitcher shaped, grows laterally, giving the first inkling of the mature appearance (Fig. 28). Tall hair cells do not change their cylindrical appearance (Fig. 9). Cytoplasmic organelles which prior to the 11th day appear randomly distributed, congregate primarily beneath the nascent cuticular plate. In the distal end of the basilar papilla, these developmental events lag behind.

The subsurface cistern is a membrane system (1–2 μm in length) that lies in parallel to the hair cell's lateral plasma membrane (Figs. 14 and 15) and is similar in appearance to the synaptic cistern. Ribosomes are usually attached to the cytoplasmic face and mitochondria congregate nearby. Although both lateral and unilateral subsurface cisterns have been identified, it is not known whether the latter is a normal occurrence or is instead due to the plane of sectioning.

In the supporting cell, the transient kinocilium disappears, the cisternal population that had collected in the apex peaks before its discharge and the neck is invaded by Golgi com-

plexes. At their bases, coiled rough endoplasmic reticula (rER) also make their appearance. The tectorial membrane reaches the climax of its formation.

16th day (Stage 42)

From the 11th–16th days (Fig. 16), the cuticular cone increases in size by growing downward and displacing the underlying organelles. Large numbers of microtubules are paraxially aligned and seem to converge on the cuticular cone. Multivesicular bodies remain closely associated with the cuticular cone.

In the supporting cells (Fig. 16), the cisternal population of rER is already being replaced by a large number of Golgi complexes. Occasionally, the basal body of the transient kinocilium is still present. The cytoplasmic density is steadily declining.

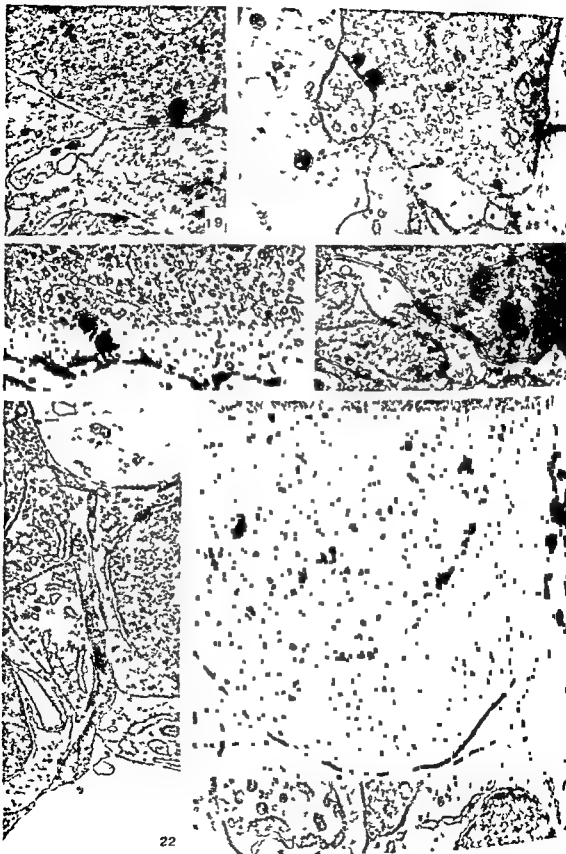
17th day (Stage 43)

In hair cells, organelles still remain beneath the developing cuticular cone. Efferent nerve fibers have not yet reached the bases of hair cells. In the bases of the supporting cells, the coiled rER is being replaced by fragmented rER, and at the apex or neck of supporting cells, Golgi are receding but are more numerous than cisternal rER.

19th day (Stage 45)

In the hair cells organelles are still congregated below the incomplete cuticular cone (Fig. 11), which now extends down as far as 2.5 μm (Table I), and the stereocilia with long rootlets reach up as much as 4.5 μm . The cuticular cone often touches the lateral membrane and points towards the inferior fibrocartilaginous plate. Occasionally, stray microtubules are embedded within the cuticular cone.

We identified as many as 4 synaptic bars in a single hair cell with multiple endings. A single afferent terminal may exhibit two synaptic bars which in some instances may be



μm apart or in others almost fused together sharing the same vesicles. Efferent terminals now begin to establish synaptic contact with hair cells (Fig. 23), but they are relatively infrequent at this time. The first subsynaptic cistern also appears concurrently with the efferent contact and directly faces it. The efferent synapse is characterized by its lateral membrane that is also filled with dense material (Fig. 24).

In the neck of the supporting cells Golgi complexes, polarized paraxially, have succeeded the majority of previous organelles. Microtubules traverse the clear cytoplasm and end at the base of the microvilli. Meanwhile, at their bases, abundant amounts of fragmented rER are now present instead of conglomerates of tubular rER, a pattern that is observed in the newly hatched chick.

10th-21st days (Stage 46) and hatching

By hatching the hair cells (Fig. 11 and 12) exhibit the same morphological characteristics as those observed in adult chickens, except that the cytoplasm is densely packed with free ribosomes. In all three hair cell types, the cuticular cones have almost reached their adult size (Fig. 17). Stereocilia, with their

rootlets anchored firmly into the cuticular cone, extend upward sometimes up to $6 \mu\text{m}$ from the short hair cells (Table I). The organelles are no longer congregated beneath the cuticular cone and are now dispersed, having lost their previous polarity. A stray mitochondrion, microtubules and vesicles are sometimes embedded within the cuticular cone (Fig. 13). Few Golgi complexes and rER are found in the supranuclear region. Up to 30 mitochondria can now be counted in a thin section, having doubled in number from earlier stages. Although some mitochondria are perinuclearly positioned, they are mainly grouped in either supra- and infranuclear regions of the hair cells, since now, the nucleus lies very close to the lateral walls of the elongated tall hair cells (Table I). The nucleus has also increased its diameter slightly (Table I).

Afferent nerve endings more frequently synapse on tall than on short hair cells (Figs. 20 and 21), the converse is true for efferent (Fig. 24). However, synaptogenesis continues at least for a short period, which accounts for the increased number of afferent and efferent terminals after hatching. Even though the afferent synapse is irregularly contoured, the pre- and post synaptic membranes remain in register and the synaptic cleft is filled with dense material. A group of axons lose their myelin sheaths as they enter through the habenula perforata (Fig. 26).

In the neck of supporting cells, the cytoplasm is clear and the organelles have almost completely disappeared except for a few Golgi complexes, which are now paraxially polarized. The kinocilium's basal body has also disappeared in most cells, though there are exceptions (Fig. 28). The supporting cell's apex is constricted to about $0.5 \mu\text{m}$, which is one-fourth the width at the 11th day. The number of satellite bodies has decreased by two-thirds, their symmetry is also lost. At their bases, fragmented rER still remains but in smaller amounts. Microtubules extend into microvillar bases (Fig. 18) but the microvilli contain microfilaments.

Fig. 19 Earliest afferent terminal on tall hair cell. Synaptic bar encircled by vesicles. Hovers over synapse. 11th day $\times 29,000$.

Fig. 20 Multiple afferent terminals on tall hair cells. There are two synaptic bars in one terminal and one in the other. 3 days after hatching $\times 19,000$.

Fig. 21 Afferent terminal in tall hair cell shortly before hatching. Two synaptic bars that are sharing vesicles face each other. 20th day $\times 19,000$.

Fig. 22 Efferent neurite penetrating into basilar papilla close to basilar membrane and not yet synapsing upon hair cells. 11th day $\times 14,000$.

Fig. 23 The first indication of an efferent synapse which is recognizable by the subsynaptic cistern. Terminal contains a few scattered vesicles. 19th day $\times 29,000$.

Fig. 24 Efferent ending. They are more frequently found on bases of short hair cells and are also usually larger. The terminal contains clear vesicles. Subsynaptic cistern is filled with a dense material and ribosomes adhere to the cytoplasmic face. 3 days after hatching $\times 24,000$.

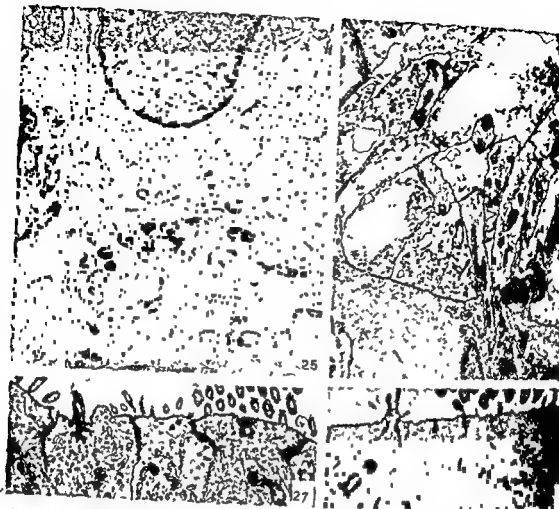


Fig. 25 Nascent basilar membrane. $\times 5000$.

Fig. 26 Habenula perforata. Axons penetrate through the hole in basilar membrane and then course tortuously between supporting cells. The basilar membrane rests on the ledge of the superior fibrocartilaginous plate. The

darker, vesiculated fibers are presumed to be cell bodies. 3 days after hatching. $\times 5000$.

Fig. 28 Diplosome in supporting cell. Diplosomes are at this developmental stage. Microvilli fan out from constricted apex. 3 days after hatching. $\times 14000$.

DISCUSSION

Differentiation of hair cells

We determined the developmental sequence of auditory hair cells in the embryonic chick at the fine structural level. The epithelial cells of the basilar papilla undergo terminal mitotic divisions between the 7th and 8th days of incubation (Fig. 1). Cellular differentiation begins immediately after the cessation of mitosis but does not occur simultaneously throughout the lagena (Fig. 6). Instead, there are two developmental gradients: the proximal portion

differentiates before the distal and the superior portion matures before the inferior; a polar pattern occurs along a basal to apical gradient in the coiled mammalian cochlea (Rubin, 1967). For example, the denser nascent hair cells first become distinguishable from the paler supporting cells between the 8th and 9th days at the superior fibrocartilaginous plate (Fig. 5), followed by the cells in the inferior fibrocartilaginous plate, as also reported earlier by Rebollo and Rodriguez (1964) and Rebollo and Casas (1964). Friedmann (1966) demonstrated that explanted otocysts, those

growing slightly, achieved equivalent development by the 12th day of culture

Under light microscopy, the basilar papilla of 11 day embryos resembles that of adults, but by the 13th day gives the impression of cellular shapes that histogenesis has largely ceased and maturation is completed (Held & Casas, 1964). However, when specimens of the same age are examined ultrastructurally, it is obvious that the nascent hair cells are actively engaged in the formation of more conspicuous specialized features, such as the stereocilia, and the cuticular cone (Figs 14, 16, 17). The three hair cell types, i.e., tall, intermediate and short, become distinguishable by the 11th day (Figs 7 and 8). Intermediate hair cells are not discussed further because they share characteristics common to both tall and short hair cells. Hirokawa (1977) was able to identify the embryonic chick's tall and short hair cells by the 14th day but apparently did not sample between the 10th–14th days when the major transformations are occurring. Held (1927) illustrated two hair cell types (pp. 493, 495) in the embryos of the chick and 16 day pigeon which he described as "zyklindrisch" and "kegelförmig" but did not mention when they first became distinguishable. Kikuchi & Hilding (1965a) demonstrated that mature outer and inner hair cells are identifiable at birth.

Kinocilia closely resemble the structure of motile cilia. They terminate in basal bodies (Figs 3 and 4) that are believed to arise from centrioles after the final mitotic divisions (Kikuchi & Hilding, 1965a). Avian kinocilia may serve a sensory function, for, along with the tallest stereocilia, they are embedded into the lower surface of the tectorial membrane. In the embryonic chick, a single kinocilium is centrally positioned in each presumptive hair and supporting cell of the undifferentiated epithelium (Fig. 1). The kinocilia are structurally identical to those in pigeons (Rosenhall, 1971, Takasaka & Smith, 1971) and young adult cocks (Janke et al., 1969) and are taller than adjoining stereocilia, unlike rudimentary

kinocilia of terminally cultured otocysts (Friedmann, 1967, 1969). In birds, kinocilia, and their basal bodies in hair cells but not supporting cells (Fig. 8) remain permanently after maturation (Takasaka & Smith, 1971). In mammals, cochlear kinocilia are transient structures that disappear shortly after hair cells mature, but their basal bodies remain (Kikuchi & Hilding, 1965a), except in the cat (Spoendlin, 1966). Vestibular kinocilia, however, are retained in adults (Engstrom et al., 1962). Although kinociliary loss is interpreted as a feature of more advanced ears (Janke et al., 1969, Rosenhall, 1971), its significance is uncertain because auditory sensitivity is seemingly unaffected.

All the stereocilia of a hair bundle begin to form concurrently but then grow at unequal rates, the stepwise differences in height are evident as early as the 11th day (Figs 7 and 8). Stereocilia are taller in tall hair cells than in short (Table I). Rosenhall (1971) has pointed out that the bird's hair cells are not arranged in rows as are mammalian outer and inner hair cells, but instead lie in a mosaic pattern. Nonetheless, stereocilia and kinocilia, when present, are morphologically polarized throughout the auditory lagena (Rosenhall, 1971, Takasaka & Smith, 1971) and cochlea (Versall & Lundquist, 1966).

The cuticular cone, a droplet of granular-amorphous protein that also contains PAS positive material (Rebollo & Rodriguez, 1964), anchors the stereocilia. The shapes of cuticular cones differ after the 14th day (Figs 9–13, 14, 16, 17). In tall hair cells, they are narrower and deeper, where in short hair cells they are wider and shallower (Table I). As the cuticular cone grows downward, stereocilia grow upward (Table I), suggesting a relationship between the two. The rootlets of tall stereocilia are longer than those of short and are anchored in the thicker portion of the cuticular cone. The stereociliary rootlets sometimes traverse the entire length of the cuticular cone (Fig. 17), but in our observations do not extend into the cytoplasm as often occurs in

mammalian cells (Kimura, 1975). Occasionally, a mitochondrion (Fig 13) is embedded within the cuticular cone.

The subsurface cistern is evident by the 14th day (Figs 14 and 15). As implied its proximity to adjoining supporting cells, it may serve as a metabolic link between them. In mammals the subsurface cisterns are highly fenestrated and face a fluid space, suggesting that they take up material. By comparison, the subsynaptic cistern faces an efferent nerve that is the same size and closely follows its contours throughout much of its extent (Fig 24). Kimura (1975) has suggested that the subsynaptic cistern may limit the spread of transmitter substance, but conversely may also facilitate efferent nerve activity. In adult mammals, these two membrane systems are directly connected, which implies that each influences the other (Kimura, 1975). Henkart et al (1976) proposed that subsurface cisterns may couple intracellular activity to electrical activity. We have not yet determined whether the subsynaptic cistern, which appears the 19th day, forms from an expanding subsurface cistern as it descends into the cell's basal end or is induced by contact of the efferent, or, once formed, attracts the efferent nerve. Hirokawa (1977) located prospective efferent contacts as early as the 14th day on the basis of subsynaptic cisterns. At present we cannot completely reconcile this temporal difference, though he may have sampled the more precocious proximal end.

In the embryonic chick, the differentiating hair cells, which display a granular cast, are considerably denser than those of the adult pigeon (Rosenhall, 1971; Takasaka & Smith, 1971) and young adult cock (Janke et al, 1969), attesting to the high level of synthetic activity, the invaginated nuclei of the differentiating epithelium (Fig 2) may presage these events. As hair cells lengthen the nuclei increase slightly in diameter (Table I), nucleoli (Figs 7-13) are more prominent than adults. In tall hair cells the nuclear membrane closely approaches the sides and in short hair cells the

basal end (Figs 7-12). As a result the cytoplasmic organelles migrate or are displaced. Sher (1971) demonstrated similar cell migrations during postnatal maturation of murine cochlea. From the 11th day after hatching, the supranuclear regions of short and tall hair cells contain an abundance of rough endoplasmic reticulum, secretory granules and several multivesicular bodies, the latter being lysosomal derivatives (Novikoff & Holtzman, 1976). In contrast other organelles steadily decline in number, especially in the infranuclear region of tall hair cells. Mitochondria, Golgi apparatuses and multivesicular bodies are rarely seen after hatching when this transition was ending. By comparison mitochondria come more numerous as hatching (21st day) nears and also shortly thereafter. The transition of organelles undoubtedly represents a transition between diminishing synthetic and emerging functional activities. Audition, however, apparently precedes structural maturation in the chick but not the mouse. For instance Vanzulli & Garcia Austt (1963) record microphonic potentials as early as the 11th day. Moreover, Grier et al (1967) demonstrated that embryonic chicks respond to sounds as early as the 12th day and this can be imprinted prenatally to particular sounds. In the murine cochlea hearing measured by Preyer's reflex, cochlear potentials and 8th nerve action potentials appear 9 to 14 days postnatally when the organ of Corti has largely completed maturation (Alt & Ruben, 1963).

Innervation

According to Knowlton (1967) neurites first intrate inside the basilar papilla as early as the 4th day, but by the 7th day, when this transition began, have not yet established synaptic contact with hair cells (Fig 25). Although afferent neurite endings first contact the hair cell bases, which are displaying slight membrane thickening by the 10th day, it is not certain whether these connections are functional. By the 11th day the neurite branches are denser, and synaptic bars the

ence of afferent synapses (Cordier, 1964a and b, Smith 1967), first appear presynaptically (Fig 19). The vesicles that surround the synaptic bars do not yet encircle them as symmetrically as they will regularly by the 13th day.

In the embryonic chick, the innervation pattern on hair cell types corresponds to that on the adult pigeon (Rosenhall, 1971, Takasaka & Smith 1971). Nerve fibers lose their myelin sheaths as they pass through the habenula perforata and advance toward the bases of the hair cells (Fig 26). Takasaka & Smith (1971) traced the course of afferent nerve fibers in pigeons and demonstrated that 90% did not branch at their terminals, only a few (2-5%) exhibit several branches. In the embryonic chick, by comparison, Fermin & Cohen (unpublished) showed that afferent nerves branch more frequently and then also arborize in the vicinity of hair cells. The hair cells usually receive more than one afferent synaptic terminal (Fig 20) but we do not know whether these multiple terminals represent mono- or polynuclear innervation. For instance, tall hair cells receive one to five afferent synaptic contacts which are usually small bouton-like terminals (Fig 21) but occasionally receive large axonomic, short hair cells receive one to two contacts which are usually large bouton-like. Friedmann (1969) demonstrated large crescentic contacts in cultured otocysts but these appear to be vestibular rather than lagenar hair cells. In the murine the synaptic contacts of the auditory lagena are restricted to the basal end of hair cells (Cordier, 1964a and b, Takasaka & Smith, 1971), whereas in the mammalian cochlea they frequently synapse laterally (Smith 1967).

Although efferent synapses may form earlier as suggested by Hirokawa (1977), we found the first convincing evidence two days prior to hatching (Fig 23), when the hair cells and associated structures of the basilar papilla are almost terminally differentiated, efferent neurites may penetrate into the basilar papilla by the 11th day (Fig 22). The efferent ter-

minals contacting tall hair cells are fewer and smaller than those synapsing upon short cells. Although they may be closely apposed, efferent nerve endings apparently do not synapse upon afferent nerves, a pattern that is common for inner but not outer hair cells of many mammals (Spoendlin, 1973).

Kikuchi & Hilding (1965a) reported that in the mouse efferent nerve fibers are first observed by the 8th-9th days postnatally when the organ of Corti closely resembles that of the adult. They (1965b) also showed by using a strain of hereditarily deaf mice (Shaker-1) that the hair cells begin to degenerate shortly after the time that efferent innervation fails to form, implying that the developing otocyst requires efferent innervation to complete differentiation and to function fully. Moreover, according to Friedmann (1959, 1965, 1969) the absence of efferent innervation may be one reason why hair cells in cultured otocysts did not complete differentiation but instead loosely resembled tall hair cells of 15-17 day embryos, the afferent nerve supply remained intact. In contrast, Van De Water (1976) reported that removal of the statoacoustic ganglion complex of murine cultured otocysts at different critical testational periods did not adversely affect differentiation. Nonetheless, it can be argued that the nerves had already imparted their influence before the excision and that in this instance afferent rather than efferent nerves are the more important. Hirokawa (1977) demonstrated that the embryonic chick's hair cells developed normally but were slightly smaller even when both the afferent and efferent nerve supplies were lethally damaged with the nerve poison *II* bungarotoxin, suggesting that hair cell differentiation, including formation of synaptic bars, synaptic membrane thickenings and densities, and cisterns does not require neural influences.

Supporting cells

During development, as hair cells become denser and acquire new structures, the supporting cells become lighter and change their

shape by transforming from long cylindrical cells to ones that are highly constricted at the apex (Fig 18) and greatly expanded at the bases, an arrangement that holds the hair cells in place and confers sufficient rigidity for stimulation. Nonetheless, there is the possibility that supporting cells serve additional functions (Siegel et al, 1977).

In birds, it is known that six supporting cells, each tightly coupled to the adjoining one, completely encircle every hair cell at its upper or apical end (Takasaka & Smith, 1971). Supporting and hair cells are also coupled to each other by tight junctions and desmosomes. Orr (1975) showed the early formation of junctional complexes in cultured chick's otocysts. The junctional complexes ensure that the endolymphatic and perilymphatic fluids, which differ markedly in composition, do not mix by flowing between the hair and supporting cells. The same junctional configuration binds hair cells and the reticular lamina in the mammalian cochlea (Iurato, 1967). Dohleman (1967) has suggested that homologous cells in the membranous labyrinth, the *plana semilunata*, may also serve a metabolic role in nourishment and maintenance of vestibular hair cells. More recently, Cohen et al (1977) demonstrated that supporting cells contain higher glycogen levels than the adjacent hair cells, which lose glycogen as differentiation nears completion. Janke et al (1969) also suggested that the supporting cells may be secretory, for they noticed that their microvilli were ensnared in the filamentous strands of the overlying tectorial membrane. This putative secretory function is strongly implied by the congregation of cellular machinery in the apices of the cells (Figs 13-18). The sacs of proteinaceous material apparently discharge their contents, for these organelles disappear after the foregoing period of intense synthetic activity. The cytoplasm then becomes clear and loses its abundant population of organelles (Figs 11-18). Kikuchi & Hilding (1965a) observed that the mouse's hair and supporting cells have transient kinocilia that are shed shortly after

birth. In the embryonic chick the kinocilia of the supporting cells are usually shed around the 14th day, but the basal bodies sometimes remain until after hatching (Fig 28).

Satellite bodies, putative complexes of nucleic acids that may be related to localized synthetic activities of microtubules (Berns et al, 1977), encircle the basal body (Figs 1 & 27) but become less numerous and smaller as hatching nears, probably corresponding to the generally diminished synthetic activities. Though present in both hair and supporting cells, they are more conspicuous in the periphery of the cytoplasm of the supporting cells.

ZUSAMMENFASSUNG

ersten Arten zeigen Am 11 Tag sind die Haarzellen Typen unzweideutig. Haare (mehrere Stereocilien) am 13 Tag gleichen sie schon denen der Erwachsenen. Der Nucleus nimmt verhältnismäßig weniger Raum ein während die Haarzellen sich vergrößern um sich schließlich im Mittelpunkt der HHZ und am unteren Teil der HHZ zu befinden. Die Nucleoli die während der Entwicklung sehr deutlich zu erkennen sind bleiben ganz erhalten. Am 11 Tag sichtbar waren wurden synaptische Berührungen und deren Zisternen erst am 19 Tag bemerkbar. Aber die Stützzenellen verwandeln sich von zylindrischen Zellen zu flaschenartigen Zellen mit verengtem Hals und diese mochten teilweise die Membrana tectoria ausscheiden.

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CSF TOTAL PROTEIN NORMAL VALUES

A Re appraisal and Discussion of its Value in Diagnosis of Acoustic Neuromas

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Abstract A reference material of total cerebrospinal fluid protein (CSF protein) from 33 men and 45 women is presented. Lowry's Folin phenol method for determining CSF protein has been used unchanged in this laboratory since 1964 with normal values ranging from 0.2 to 0.4 g/l.

Determining CSF protein in diagnosis of acoustic neuroma. The results showed both statistically as well as clinically significant increased protein, but these tumours can be easily diagnosed by other means. A more detailed determination of CSF protein is discussed.

In 1951 Lowry et al published their study on the measurement of very low concentrations of proteins with the use of Folin and Ciocalteu's phenol reagent after pre-treatment of the protein with an alkaline copper solution. Since then this method, or modifications of it, under names as the Lowry-, the Folin-Lowry, the Folin-Lowry-Ciocalteu's method etc. has been widely accepted for determination of proteins in dilute fluids. In 1956 Lous et al. after a careful evaluation of the method for determination of total protein in cerebrospinal fluid (CSF) recommended it as "a simple, accurate and highly satisfactory routine method". The method was introduced in this department of clinical chemistry in 1964 and has been used unchanged since then. At the time of its introduction no available normal values for the method existed and therefore the values 0.20-

0.40 g/l based on an earlier turbidimetric method (Merritt & Fremont Smith, 1938) were used.

Due to the lack of a reference material of our own, and due to an increasing feeling from the clinicians that the reference values were too low (Björum et al., 1972), the collection of a reference material was undertaken.

MATERIAL

98 persons, 53 men and 45 women, in the age range 18-72 years, were included, 75 were psychiatric patients and 23 orthopedic patients who, due to minor disorders, were to be operated upon under spinal anaesthesia. The patients were neurologically and medically normal according to the recommendations of The Committee of Reference Values of The Scandinavian Society for Clinical Chemistry and Clinical Physiology (1975). None of the patients received medication in the form of streptomycin, sulfanilamide, salicylates, acetylsalicylic acid, phenacetin or psychotropic drugs apart from diazepam 10-20 mg/day, which were given as a sedative or hypnotic, when indicated.

Lumbar puncture technique

The lumbar puncture was performed between 8 and 9 a.m. after the patients had been kept fasting and recumbent in bed for the preceding 10 hours. The patients were placed in

Table I Comparison of the present Folin-Lowry method with the biuret method

Specimen	Dilution	Protein concentration g x l ⁻¹		
		Biuret method		Folin-Lowry method
		Measured	Calculated	Measured
Seronorm® batch 127	undiluted	67		
	1 100		0.67	0.72
	1 200		0.34	0.41
	1 333		0.20	0.23
Serum pool	undiluted	69		
	1 100		0.69	0.72
	1 200		0.35	0.40
	1 333		0.21	0.26

lateral position and local anaesthesia was induced by lidocaine norepinephrine. A CSF specimen of totally 10 ml was obtained by fractionated sampling. Only clear and colourless CSF was used for analysis (Bech et al, 1978).

Protein determination

The modification of the Lowry method used for protein determination is that of Daughaday et al (1952) scaled down to use only half the volumes.

Lous et al (1956) and Cannon et al (1974) have described and evaluated this method in detail, and only the major principles are described here.

Two reactions are involved, (1) by addition of Cu²⁺ ions in alkaline solution the protein is complexed as in the biuret reaction. (2) The Cu protein complex reduces the added Folin-Ciocalteu reagent (polyphosphomolybdic and polyphosphotungstic acid) to the deeply coloured molybdenum- and tungsten blue. In proteins—especially the amino acids—tyrosine and tryptophan give rise to an extra staining, adding to that produced by the Cu-protein complex. Certain drugs such as streptomycin, salicylates, acetylic salicylic acid, phenacetin and chlorpromazine also cause staining. Moreover it has been shown that IgG exhibit

20% higher absorbance (on a mass basis) in the Folin-Lowry method than does albumin (Rieder, 1974, Cannon et al, 1974). Because of the variation in chromogenicity of various proteins, the use of diluted serum as standard for measuring CSF proteins as proposed by Cannon et al (1974) will standardize the error due to this difference.

Double determinations were performed on 200 µl CSF taken from the third ml CSF. As standard was used a serum pool with a total protein mass concentration of 70 g/l determined by the Kjeldahl method diluted 1/100 with 154 NaCl to 0.70 g/l. No corrections for non protein material, according to Daughaday et al (1952) 0.06 g/l, was made.

The analytical variation (precision) between days shows a standard deviation of 0.010 g/l. The samples were in the range 0.20–1.20 g/l with a mean value of 0.49 g/l. This gives a coefficient of variation of 2%.

In an attempt to estimate the analytical bias (the accuracy) of the method as it is run in this laboratory, a short re-evaluation was performed in connection with this study. Ten protein solutions used as controls in the serum protein determination was analysed in dilutions of 1/100, 1/200 and 1/333. It was the Seronorm® batch no. 127 with a protein con-

Table II Protein concentration of orthotic spinal fluid control (lot 4p154) values of the present Folin-Lowry method compared with the declared values

Method	Protein concentration g x l ⁻¹
	Declared values (x ± 2 s)
Turbidimetry trichloroacetic acid*	0.80 ± 0.12
Turbidimetry trichloroacetic acid*	0.85 ± 0.15
	Measured value (x ± 2 s)
Folin-Lowry (present method)	0.82 ± 0.04

- * Dupont aca®
- * Henry et al (1956)

Table III CSF total protein in a reference material of 98 persons grouped according to sex and age

Sex	Age (years)	Number of subjects	CSF protein g \times l ⁻¹		Signif. of diff.
			Median	Range	
♀	18-40	17	0.39	0.23-0.69	P 0.02
♂	18-40	27	0.50	0.26-0.96	
♀♂	18-40	44	0.44	0.23-0.96	
♀	40-59	19	0.43	0.28-1.06	P<0.05
♂	40-59	18	0.53	0.35-0.98	
♀♂	40-59	37	0.49	0.28-1.06	
♀	60-72	9	0.45	0.31-0.70	P 0.11
♂	60-63	11	0.53	0.41-0.87	
♀♂	60-72	17	0.49	0.31-0.87	
♀	18-72	45	0.42	0.23-1.06	P 0.001
♂	18-63	53	0.53	0.26-0.98	
♀♂	18-72	98	0.47	0.23-1.06	

centration of 67 g/l, and (2) a pooled serum, used as control on the Autoanalyser® SMA 6/60 with a protein concentration of 69 g/l, both determined by the biuret reaction. This method is standardized with human serum albumin from AB Kabi, Stockholm. The nitrogen content in this albumin is determined and protein concentration calculated (N-content taken to be 16%).

Further in a 2 week period, Ortho® Spinal Fluid Control, lot no. 4P154 was analysed. According to "assay data" supplied with the control the expected protein concentration was 0.80 g/l \pm 0.12 g/l as determined on ACA® (Automatic Clinical Analyser, DuPont Company, Instrument Product Division, Wilmington, Delaware) with a turbidimetric method using trichloroacetic acid or 0.85 g/l \pm 0.15 g/l determined with the turbidimetric method of Henry et al. (1956) also using trichloroacetic acid.

RESULTS

The results of reevaluating the method is seen from Tables I and II. The comparison between the biuret and the Folin-Lowry method in Table I shows a slightly higher estimation of the protein concentration by the last mentioned method. As has been stated by Cannon

et al. among others (1974) the photometric determination does not strictly follow Beer's Law, i.e. there is a slight deviation from the linear correlation between calculated protein concentrations in dilutions and the measured. As a consequence, protein concentrations below the concentrations of the standard used (0.70 g/l) are overestimated, and concentrations above are underestimated, in both cases it is less than 5%. Specimens with concentrations above 1.0 g/l are to be diluted and reanalysed. So this observation has only little practical and quantitative importance.

The measurements on the commercial CSF-control in Table II show good agreement with the values reported.

Table III shows the results of protein determinations in CSF arranged in groups according to sex and age. Males exhibit significantly higher values ($p=0.001$) than females. No correlation existed between age and CSF protein concentration (Spearman rank correlation test).

No difference between the psychiatric and orthopaedic patients could be shown (by the Mann-Whitney test).

Figs. 1 and 2 display the CSF protein distribution among females and males, Fig. 3 the distribution in the total material.

For practical purposes there is no reason to

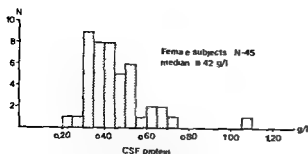


Fig 1 CSF protein in 45 female subjects

make distinctions between males and females as to the protein concentrations in CSF

The 0.05–0.95 fractile interval in the present total material is 0.29–0.88 g/l, which now is used as reference interval for this laboratory

DISCUSSION

Estimating the analytical bias (the accuracy) of the method in determining the protein concentration in heterogeneous protein solutions as CSF (and serum) has certain shortcomings as the actual concentration of protein components with varying chromogenicity in the actual sample is unknown

It has been claimed that the results are relatively unaffected by changes in the albumin/globulin ratio. Results have also been reported to agree well with the total proteins determined by Kjeldahl analysis for normal CSF and diluted serum (Lous et al, 1956, Cannon et al, 1974)

The results of re-evaluating the methods as mentioned above exhibit an acceptably low analytical bias for quantitating total protein concentration in CSF

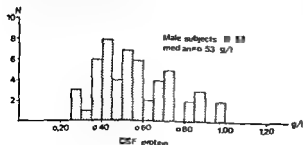


Fig 2 CSF protein in 53 male subjects

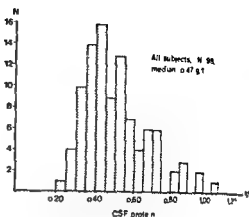


Fig 3 CSF protein in 98 subjects the total reference material

The results have shown a sex difference in CSF protein concentration, with higher values among men compared with women. The difference is significant for the age groups up to 59 years, and for the total material. This is in contrast to the findings of Jung et al (1973) and Tibbling et al (1977). In contrast to these authors we were unable to register any significant correlation between CSF protein concentration and age. These authors did not attach any practical importance of their findings and neither did we find any reason to make distinctions between males and females for practical purposes as to the protein concentration in CSF.

A 0.05–0.95 fractile interval of CSF concentration at 0.29–0.88 g/l with a median value of 0.47 g/l is one of the highest reference values found in the literature. Tibbling et al (1977) found in 93 subjects a mean value of 0.43 g/l and a range of 0.27–0.80 g/l (estimated from figures) and Jung et al (1973) in 11 subjects, presented a normal range of 0.16–0.41 g/l ($\bar{x} \pm 2s$).

The discrepancies in the CSF protein concentrations found in the literature (Jung et al, 1973, Tibbling et al, 1977) may depend on the selection of the subjects included and the methods used for analyses and various standards adopted, e.g. diluted serum albumin, tyrosine or mixtures of tyrosine and albumin as mentioned by Tibbling et al (1977). Jung et al

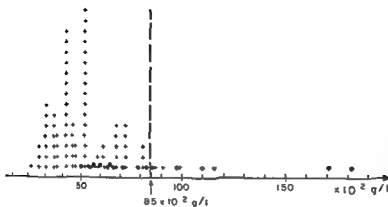


Fig 4 CSF protein in reference material (+) in relation to CSF protein in 16 patients with medium sized acoustic neuromas (●)

73) mention a possible geographical influence on the CSF protein concentration. Our results confirmed the clinical impression that our previous reference values for F protein were too low.

clinical implications

It has previously been reported by Thomsen (1976) that among medium sized and large acoustic neuromas the majority if not all had increased CSF protein, using an upper limit of 100 g/l for the normal values. This has also

been reported by Pulec et al (1971), Fisch & Wegmüller (1974), Ojeman et al (1972), among others. We therefore compared our previous CSF protein concentrations in operatively verified acoustic neuromas with the new reference material (Figs 4 and 5). The neuromas were 16 medium sized, in 10 female and 6 male patients, aged 25–67 years, and 61 large tumours, among 39 female and 22 male patients, aged 19–72 years. Age and sexwise, they did not differ from the reference material. Even though there is a statistically significant

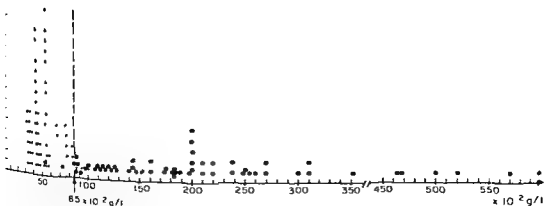


Fig 5 CSF protein in reference material (+) in relation to CSF protein in 61 patients with large acoustic neuromas (●)

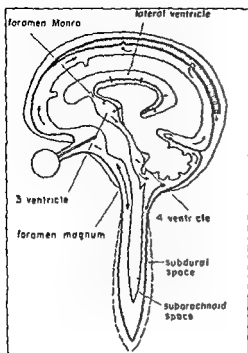


Fig 6 Schematic drawing of CSF flow

difference in total CSF protein between the reference material and patients with medium sized tumours ($p < 0.001$), and between patients with medium sized and large tumours ($p < 0.001$), using the Mann-Whitney test, there is so much overlapping among the patients with medium sized tumours and the reference group that only 37% exhibit abnormal CSF protein concentration, applying an upper limit of 0.85 g/l. This indicates that the value of determining CSF protein in patients with medium sized tumours is most questionable. Thus the difference between the reference group and the group of patients with medium sized tumours has only statistical but no clinical significance. In contrast to this the difference between the reference group and the group of patients with large tumours has both statistical as well as clinical significance. However, with a large tumour the patient will exhibit a bulk of other symptoms, and the diagnosis is easily made by other means (Thomsen et al, 1977). We therefore feel that the determination of CSF protein in the diagnosis of acoustic neuromas is of little if any value.

There is an estimated daily production of 600–800 ml cerebrospinal fluid. It is produced in the choroid plexus by ultrafiltration of plasma, and the circulation is indicated in Fig 6 through the ventricular system and from the 4th ventricle to the subarachnoid space through the foramen Monro and foramina Luschkae. The absorption or drainage of the cerebrospinal fluid is believed to take place in the large sinuses, as is also seen in Fig 6. Normal CSF proteins consist in 80% of proteins from plasma, the remaining 20% is produced locally in the subarachnoid space consisting of prealbumin, transferrin, lysozyme, β -trace, γ trace, and, in local infections or autoimmune disease, immunoglobulins. The amount of CSF protein increases by increased blood-CSF barrier permeability and by leakage of parts of the liquor space (Schulze & Heremans, 1966).

Since the CSF protein elevation is mainly found in large acoustic neuromas it is most likely that the increase is due to a blockage of the circulation in the basal cisterns. It is not clear, however, whether an acoustic neuroma also gives rise to a breakdown of the blood-brain-liquor barrier, or incurs a local production of proteins. Barrier permeability is examined by determining CSF protein, CSF albumin, or better CSF albumin/serum albumin ratio. If the concentration of a protein in the spinal fluid increases due to local production the concentration must be compared with the barrier permeability. This can be done by an index calculation as described by Bock (1977), Hansen et al (1977) and Tilling et al (1977). If spinal fluid proteins are to be of diagnostic value in acoustic neuromas we feel that the determination should be supplemented by a more detailed examination of the protein content as described briefly above. Such investigations are in progress.

ZUSAMMENFASSUNG

Aus einem neuen Normalkollektiv von 53 Männern und 45 Frauen werden die Werte des Gesamteiwisses (GEG) im Liquor Cerebrospinalis vorgelegt. Seit 19

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- des Normalbereiches auf 0.88 g/l verlegt werden muß. Dieses macht die Bedeutung des GEG in der Acusticusneurodiagnose äußerst fraglich. Unter den Tumoren mittlerer Größe waren Fälle mit statistisch signifikant erhöhten GEG Werten jedoch ohne klinische Signifikanz. Die großen Tumoren zeigten zwar sowohl statistisch als auch klinisch signifikant erhöhte GEG Werte, aber diese Tumoren lassen sich leicht in anderer Weise diagnostizieren. Eine differenzierte Bestimmung des GEG Wertes im Liquor Cerebrospinalis wird besprochen.
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EVOKED POTENTIAL CORRELATES OF GENETIC PROGRESSIVE HEARING LOSS

Age-related Changes from the Ear to the Inferior Colliculus of C57BL/6 and CBA/J Mice

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Abstract Volume-averaged auditory evoked potentials (AEPs) were simultaneously recorded from the auditory nerve and the inferior colliculus (IC) of C57BL/6 and CBA/J mice. The AEPs displays genetic differences in the pattern of age-related progressive hearing loss. At 30 days post partum, amplitudes of the summating potential (SP) and cochlear microphonic (CM) were lower in the C57BL/6 genotype and they decreased progressively with age.

at the auditory nerve

for genetic progressive hearing loss in the C57BL/6 mouse. The present paper describes the application of computer averaging techniques to this animal model. Measures of electrical activity from peripheral and CNS auditory structures were non-traumatically obtained from the C57BL/6 mouse which support and extend the behavioral and anatomical evidence of Mikaelian et al. (1974).

Electrococchleography utilizes electrodes placed at the promontory or external auditory meatus to non-traumatically record inner ear electrical activity (Yoshie, Ohashi & Suzuki, 1967; Sohmer & Feinmesser, 1967; Aran & LeBert, 1968). The same principle (i.e. computer-averaging a large number of volume-conducted evoked potentials) has been applied by Jewett (1970) to the auditory brain stem, and responses have been obtained which correspond to identified regions from the cochlear nucleus to the inferior colliculus (Jewett, 1970; Jewett & Romano, 1972; Baskwald & Huang, 1975). The present experiments simultaneously measured these peripheral and CNS auditory responses in genetically defective C57BL/6 (Mikaelian et al. 1974) and normal hearing CBA/J (Finck & Berlin, 1965; Mikaelian et al., 1965; Starr, 1971; Webster & Webster, 1977) mice from 30 to 200 days post partum, relating the results to experimental and clinical literature.

The aging process in humans is often associated with a progressive sensorineural hearing loss, which has been related to both genetic and environmental factors (Schuknecht, 1974; Konigsmark & Gorlin, 1976; Hawkins, 1973). The pathology is typically described in reference to the inner ear, although auditory central nervous system (CNS) involvement has been reported in some cases of age-related hearing loss (Schuknecht, 1974; Bredberg, 1968). Although 10 to 15% of the adult population of the United States is significantly affected by hearing loss (Paparella, Hanson, Rao & Ulvestad, 1975), practical and ethical considerations make it difficult to experimentally evaluate genetic, environmental, peripheral and CNS variables. Adequate animal models would be useful for experimentally isolating these variables.

Mikaelian, Warfield & Norris (1974) have described behavioral and anatomical evidence

METHOD

of the highly inbred CS7BL/6 and CBA/J strains were used for this study. The subjects of the former strain were the 8th to 10th generation and the mice of the latter strain were the 1st and 3rd generation of parental stock obtained from the Jackson Laboratory. Only male mice were tested, to eliminate possible sex differences. The animals were bred, reared and maintained in a quiet mouse colony with the exception of that produced by the subject themselves, typically did not exceed 20 dB SPL at frequencies above 10 kHz and were given no antibiotics, to reduce environmental influences on hearing loss. From 8 to 11 mice of each genotype were tested at 50, 100 and 200 days of age. Some mice were tested at a single age, while others were tested at 2 or 3 ages. No effects of repeated testing were observed.

The subjects were anesthetized with 60 mg/kg pentobarbital, i.p., and maintained at a temperature of $37.5 (\pm 0.1)^{\circ}\text{C}$, as measured with a microthermistor placed against the right tibia. A modified version of the method of Jewett & Romano (1972) was used to obtain volume conducted responses from the right ear and from subsequent auditory brainstem regions. Electrical activity was recorded between a scalp vertex stainless steel electrode (positive) and a steel bar pressed against the soft palate (negative). The vertex electrode was especially sensitive to activity generated in the brain stem while the mouth electrode emphasized responses from the ear (Plantz, Williston & Jewett, 1974).

Acoustic stimuli were generated by amplified 0.1 msec duration square waves, transduced by an Altec 801 tweeter, and delivered by a sound tube to the right ear of the subject. At the level of the ear, the stimuli had their greatest energy at 2 kHz, with a 25 dB drop at 1 k and 10 kHz. Clicks were presented at the rate of 20 per second at SPL's from 40 to 100 dB in 20 dB steps (all SPL's are re 0.0002 dynes/cm²). The bioelectrical signals were

amplified by a factor of 10^6 with two adjacent cascaded Grass P15 preamplifiers with filter settings of 300 and 3000 Hz. They were recorded with a digital computer signal averager with an A to D conversion of 9 bits, at a temporal resolution of 10 to 30 μsec per address. The evoked potentials were averaged from the responses to 512 clicks of alternating polarity. At one SPL (80 dB), the responses to rarefaction and condensation clicks were analysed separately, but they were combined for all other measures. All averaged responses were plotted, and latency values were directly obtained from the computer and transcribed. A train of 2 kHz sine waves of 10 μV amplitude was used for system calibration. Amplitudes of inner ear responses were measured from the pre stimulation electrical value to the peak of the evoked potential. Brainstem evoked potential amplitudes were measured from the negative trough to the following positive peak. Unless otherwise stated, *t* tests were used to evaluate these latency and amplitude measures.

RESULTS

A typical volume conducted auditory response is seen in Fig. 1. The summating potential (SP) is clearly visible, although alteration of the stimulus polarity has cancelled out the cochlear microphonic (CM). Previous experiments (K. R. H., in preparation) have shown that the SP and CM correspond to the summating potential and cochlear microphonic measured from the round window. P_1 corresponds to the AP as measured from the round window and across the auditory nerve of the stimulated ear of the mouse. The dual P_1 peaks correspond to high (P_{1H}) and low (P_{1L}) intensity threshold portions of the AP amplitude input-output function. The brainstem components in the mouse have also been examined by local recording and lesion experiments (K. R. H., in preparation) and found to correspond with responses obtained from the cat (Jewett, 1970; Buchwald & Huang, 1975). The

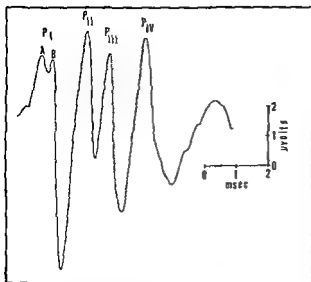


Fig 1 Volume-conducted auditory nerve (P_I) and brain stem (P_{IV}) evoked potential from the laboratory mouse (see text for explanation)

responses in the mouse and cat are generated, primarily or exclusively, at or near the following structures: P_{II} , the cochlear nucleus ipsilateral to the stimulated ear, P_{III} , the contralateral superior olivary complex, P_{IV} , the nucleus of the lateral lemniscus on both sides of the brain stem, and P_V , the contralateral inferior colliculus.

At 50 days post partum, there were no genotypic differences in amplitudes or latencies of the P_{I-V} evoked potentials at any SPL. Even though these neural values were the same, sensory measures differed between the two strains of mice. The CM in response to a rarefaction click was 55.4% smaller in amplitude in the C57BL/6 than in the CBA/J mouse ($p < 0.001$), the condensation CM of the C57BL/6 was 48.3% ($p < 0.02$) and its SP was 43.5% ($p < 0.005$) of those seen in the CBA/J strain.

By 100 days, the C57BL/6 mouse exhibited a change in function of the auditory nerve at high SPL's. P_{IV} was only seen at 100 dB in 2 of the 10 mice of this genotype, although it was recorded in 9 of the 10 100-day old CBA/J's (chi square $p < 0.005$). This situation remained unchanged at 200 days with 2 of the 8 C57BL/6 and 9 of the 10 CBA/J mice showing

P_{IV} in response to 100 dB clicks (chi square $p < 0.01$).

The neural (P_{I-V}) response amplitudes of the C57BL/6 mouse progressively decrease with increasing age. Since there were individual differences in the peak of the input-output functions (i.e., responses maximal in some subjects at 100 dB and others peaked at 80 dB) amplitudes were compared at 60 dB. Over the 150-day span of this experiment, the C57BL/6 P_I amplitude decreased by 72% ($p < 0.001$), P_{II} declined by 69% ($p < 0.0001$), P_{III} by 71% ($p < 0.001$), P_{IV} by 55% ($p < 0.001$), and P_V by ($p < 0.10$).

Although the CBA/J neural response amplitudes to this same 60 dB click were variant from 50 to 100 days, decrement was observed at 200 days of age. By this age P_I amplitude decreases by 53% ($p < 0.001$), P_{II} by 41% ($p < 0.0001$), P_{III} by 41% ($p < 0.001$), P_{IV} by 56% ($p < 0.0001$) and P_V by 51% ($p < 0.001$).

We did not observe any significant age-related latency changes in these genotypes which corresponded to the amplitude measurements at 60 dB described above. The SP and P_I latencies in response to a 60 dB click remained relatively constant over time.

By 200 days, the sensory indices at 60 dB had decreased in amplitude for both genotypes. For the CBA/J mice, the rarefaction CM decreased by 58% ($p < 0.001$), the condensation CM, by 40% ($p < 0.001$) and the SP by 54% ($p < 0.005$). These responses were small to be measured in all of the 200-day C57BL/6 mice. Therefore, the sensory decrement seems to be more pronounced than the neural decrement in the older C57BL/6 whereas sensory and neural losses appear to be approximately equal in the 200-day CBA/J genotype.

In addition to these comparisons at 60 dB SPL, each genotype was compared for changes to clicks of increasing SPL. It is reasoned that rates of amplitude and latency changes over this intensity range could

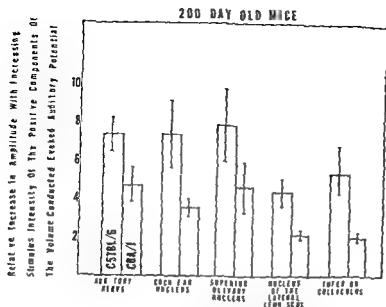


Fig 4 Same as Fig 2 at 200 days postpartum

age. By 100 days, this pattern begins to change (Fig 3). At this time, the C57BL/6 has a smaller dynamic range for the amplitude of EP from the cochlear nucleus ($p < 0.01$).

By 200 days of age, however, pronounced genotypic differences were observed (Fig 4) in the rate of amplitude change to increasing click intensity. The C57BL/6 developed a "recruitment" profile which is especially pronounced (in comparison with either 200 day-old CBA/J or with 50 day-old C57BL/6 mice) at higher brainstem levels. Examination of the raw amplitudes of each subject indicated that

these changed C57BL/6 amplitude ratios at 200 days were due to both an increase at high SPL's and a decrease at low SPL's and were not an artifact of the scaling technique. The CBA/J showed no significant change in amplitude growth functions from 50 to 200 days of age.

Even more pronounced CNS latency changes were observed as a function of age and genotype. At 50 days of age (Fig 5) there were no genotypic latency differences and the latency decrease with increasing stimulus intensity was the same at all auditory levels. At

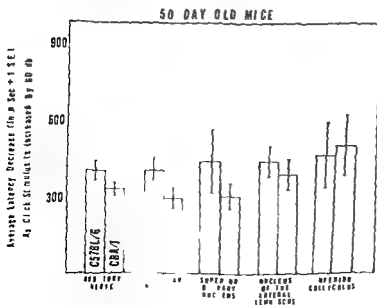


Fig 5 Relative latency changes as a function of stimulus intensity in C57BL/6 and CBA/J strains of mouse at 50 days postpartum (see text for explanation)

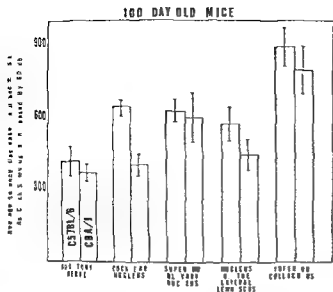


Fig 6 Same as Fig 5 at 100 days post partum

both genotypes aged, greater latency changes were observed in central than in peripheral structures, but there were still no genotypic differences at 100 days except in respect to activity from the cochlear nucleus ($p < 0.001$, see Fig 6). By 200 days, however, there were major genotypic differences for CNS (but not peripheral) auditory regions. At this older age, C57BL/6 mice have more pronounced latency decreases for $P_{n IV}$ than do CBA/J mice (Fig 7). This CNS effect was primarily due to an increase of latencies at 40 dB, with normal latencies being seen at high SPL's.

DISCUSSION

The data of the present study support the behavioral and histological data of Mikaelian et al (1974), and their conclusion that the C57BL/6 mouse develops progressive hearing loss early in its life. When sensory measurements (CM and SP) were made at 50 days of age, the values of the C57BL/6 were significantly lower than those of the CBA/J genotype. This experiment also compliments the earlier behavioral audiograms with the inclusion of suprathreshold amplitude and latency functions, as well as measures of electro-

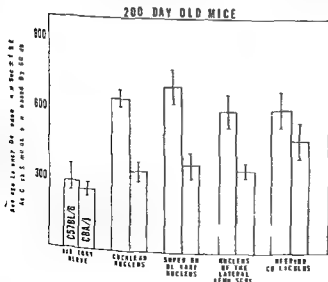


Fig 7 Same as Fig 5 at 200 days post partum

physiological changes which progress from the auditory periphery to higher brainstem levels

The electrophysiological deficits of the C57BL/6 mouse were first observed at puberty (50 days of age), slightly earlier than the age at which Mikaelian et al (1974) reported behavioral high frequency hearing loss, and considerably earlier than the age at which they first observed inner ear pathology in this subject. The CM of the senile guinea pig was also found to be reduced in amplitude (Pestalozza et al, 1957). Crowley et al (1972a) reported similar CM decrements in the 24 month old rat. To our knowledge, the SP has not yet been examined in the rodent with age related hearing loss.

The auditory nerve action potential (AP, or P_0) of the C57BL/6 declined in amplitude within 50 days of the observed CM loss. Crowley et al (1972b) also reported smaller AP's in the aged rat. Bergholtz, Hooper and Mehta (1977), using transtympanic electrodes in the presbycusic human, noted a tendency for smaller AP's to be seen in subjects with increasing hearing loss.

The waveform of the compound AP has been correlated with specific auditory dysfunctions (Aran, 1973; Eggermont, 1976; Yoshie, 1973). Bergholtz et al (1977) have associated certain AP waveforms with the presbycusic human, but feel that this presents only a crude estimate of cochlear damage. The human AP obtained by transtympanic electrocochleography typically shows dual peaks, with the first mode being a short latency, high (H) intensity threshold response, while the second mode has a longer latency and a low (L) intensity threshold (Aran, 1973; Eggermont & Odenthal, 1974; Eiberling & Salomon, 1976). P_{10} and P_{10} amplitude input-output curves from the mouse appear to correspond to the H- and L-curves of the human (Henry, in preparation). The observation that the aging C57BL/6 mouse is less likely to display P_{10} at higher SPL's might indicate damage to those elements which contribute to the L curve. Possible candidates include outer hair cells

and the "ups" of the frequency-threshold characteristic curves of single auditory nerve fibers (Eggermont & Odenthal 1974; Evans 1975).

It has recently been suggested that the common practice of combining rarefaction and condensation responses can distort the waveform. Coats & Martin (1977) noted that in the human with high frequency hearing loss the condensation and rarefaction AP's were quite different in appearance. This was not found to be the case with one of the mice, so it is unlikely that the results are artifacts of this procedure.

The only dynamic brainstem changes in 100 day old C57BL/6 mouse were found in cochlear nucleus (Figs 3 and 6). As stimulus intensity was increased, the cochlear nucleus (CN) evoked responses showed a significantly smaller amplitude increase than the corresponding CBA/J curves. This may be related to the selective vulnerability of the CN (in relation to other auditory brainstem nuclei) to metabolic (Fisch, 1970; Dublin 1976) or to the maturity (11 to 15 days post partum) of the nucleus in the mouse (Mionymy 1967). Congenitally sensorineural deaf mouse deprived of sound from birth also shows neural changes which are localized to the brainstem area (Webster & Webster 1977).

The 200-day-old C57BL/6 mouse shows "recruitment" of central (but not peripheral) auditory evoked potentials (EP's) relative to its 50-day old self, i.e. the EP amplitude grows relatively larger with increasing stimulus intensity in the older C57BL/6 mouse. The CBA/J mouse has no relative amplitude changes over this same age span. This "recruiting profile" has been observed in the input-output function associated with recruitment of loudness (Yoshie 1968; Aran 1973). If the older C57BL/6 mouse displays loudness recruitment, these data would suggest that reduced input to the cochlear nucleus might be involved in the process.

The CNS changes of the aging C57BL

mouse were not uniform at all neural levels. The decline of the CN and superior olivary complex (SOC) EP amplitudes mimicked that of the AP (69–72%) while a smaller (49–55%) decrease was observed at higher neural levels. This resembles the difference in P_1 (AP) and P_2 (upper brainstem) EP amplitude decrement in the mouse (Sohmer & Pratt (1975) described in humans subsequent to noise exposure).

Although the CBA/J mouse has been considered as having normal hearing (Finck & Berlin 1965, Mikaelian et al., 1965, Mikaelian, 1966, Birch et al., 1968, Sher, 1971, Webster & Webster, 1977), decrements were observed in all the electrophysiological measures by 200 days of age. The relatively uniform pattern of this loss suggests one of two possibilities: 1) CBA/Js develop a slight hearing loss which is sensory (or possibly conductive) in nature by 60 days of age (it may increase at later ages) and CNS amplitude changes merely reflect this loss; 2) CBA/J mice have no hearing loss, and these absolute amplitude changes merely reflect impedance changes in an aging mouse. This interpretation does not apply to C57BL/6 mice because they have changes which differ at various neural loci, and also show relative changes with increasing stimulus intensity (where impedance values remain constant). The relative minor age related changes of the CBA/J are in contrast to the progressive changes of the C57BL/6 mouse, in which sensory deficits during adolescence were followed within 50 days by functional changes of the auditory nerve and cochlear nucleus, with higher auditory CNS functional alterations becoming manifest at 200 days post partum.

ZUSAMMENFASSUNG

Elektrische Antworten des Hirnstamms wurden an Mäusen im Bereich zwischen Ohr und innerem Cochlea mittels äußerer Elektroden gleichzeitig gemessen. Die Maus C57BL/6, die einen genetisch verursachten sensorischen zunehmenden Gehörverlust aufweist, zeigte eine Abnahme der EP-Amplituden. Diese Abnahme war am oberen Hirnstamm (SOC) und am CN (superior olivary complex) am stärksten. Diese Abnahme ähnelt der Abnahme der AP-Amplitude (69–72%) während eine kleinere Abnahme (49–55%) an höheren neuralen Ebenen beobachtet wurde. Dies ähnelt der Differenz zwischen P_1 (AP) und P_2 (upper brainstem) EP-Amplitudenabnahme beim Menschen (Sohmer & Pratt (1975) beschrieben bei Menschen nach Lärmexposition).

fortschreitendem Alter zunehmend ab. Weitere 50 Tage später wurden in den Antworten des Gehörnervs und des cochleären Kerns derselben Maus Veränderungen festgestellt. Im Alter von 200 Tagen wies die Maus C57BL/6 in den CNS-Bereichen jedoch nicht am Gehörnerv Anzeichen von Erholung auf.

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SINGLE CELL LAYER MEMBRANE COVERING THE DEGENERATED COCHLEAR DUCT AFTER PERILYMPHATIC PERFUSION OF STREPTOMYCIN

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Abstract Ultrastructural changes of the extrasensory

of the outer sulcus cells and finally involved other
thelial cells such as Claudius and the inner and outer
cus cells. In the final stage except for the stria vas-
ana and Reissner's membrane all epithelial cells which
ed the cochlear duct were replaced by a single-cell layer
mbrae which originated medianly from the epithelial
ls of Reissner's membrane and laterally from the super-
al outer sulcus cells.

Recently, the function of epithelial cells lining
the cochlear duct have been discussed by
any investigators. One can classify these
epithelial cells into organ of Corti, stria vas-
cularis, spiral prominence cells, outer sulcus
cells, interdental cells, Reissner's membrane
cells and presumably each of these cell groups
as a certain functional role as well as func-
tioning as a perilymph barrier.

The purpose of this study was to investigate
electronmicroscopically the fate of these cell
groups after perilymphatic perfusion of oto-
toxic drugs. Despite many reports about
changes in the organ of Corti after administra-
tion of ototoxic drugs, the pathologic changes
of extrasensory epithelium have scarcely been
mentioned. In this study, we concentrated our
attention on the pathological changes of the
extrasensory epithelium. We found that the
lining of the cochlear duct—except for stria
vascularis—is ultimately replaced by a single-

cell membrane similar to that in reported
temporal bone pathologies (Lindsay et al,
1960, Nager, 1952, Beal et al, 1967). The
origin of this single cell layer membrane, not
previously reported, became clear in our
study.

We have already described the changes in
the nerve elements of the organ of Corti of the
same animals as used in the present investiga-
tion (Terayama et al, 1977). We will report the
details of the changes in stria vascularis sepa-
rately.

MATERIALS AND METHODS

Identical materials and methods as described
in detail in our previous paper (Terayama et
al, 1977) were employed for this study.

In group A, we perfused 23 cochleae of 17
guinea pigs with 20% combined streptomycin
solution (SM solution) through the scala
tympani, and the animals were sacrificed after
3 to 137 days. In group B, 8 cochleae of 4
animals were perfused with 2% SM solution
and the animals were sacrificed after 31 to 76
days. In group C, 8 cochleae of 7 animals were
perfused with Ringer's solution and the ani-
mals were sacrificed after 7 to 137 days. The
cochleae were prepared for electron micro-
scopy.

In this paper, we obtained the results mainly
from the 1st and 2nd turns of the cochlea.



Fig 1 5 days after perfusion 20% SM Organ of Corti is collapsed due to distortion of supporting cells Hair cells are lost Inner sulcus cells and Claudius cells still appear normal The scale of the electron micrographs is indicated by a black bar with number, which represents μm

FINDINGS

(1) Group A (perfused with 20% SM solution)

In this group, the degenerative changes of the epithelial cells lining the cochlear duct—with the exception of Reissner's membrane—were outstanding The degree and extent of these pathologic changes increased with the time after the perfusion We classified them into early and later stage We found no rupture on Reissner's membrane On the lateral wall, all of three layers of the stria vascularis showed characteristic pathologic changes We will report the details of their changes in another paper

(a) *Changes in the early stage* (3 to 6 days after the perfusion) In the organ of Corti, hair cells had already disappeared The tunnel space was filled with the distorted and swollen supporting cells Claudius cells and inner sulcus cells appeared normal under the light microscope (Fig 1)

The spiral prominence epithelium appeared almost normal except that many filaments were conspicuous in their cytoplasm (Fig 2A)

The outer sulcus cells in 5 and 6-day specimens showed atrophy and degeneration and

their cytoplasmic extensions (roots) had disappeared The space previously occupied by the roots became vacant except for some remaining debris In the cytoplasm of the cells underlying the superficial cells of outer sulcus and having direct contact with the degenerated roots, lysosome, vacuolization formation of lamellated bodies, and lipofuscin granules increased in number, and mitochondria increased The superficial cells of the outer sulcus showed atrophic dark cytoplasm with fewer degenerative signs than in the underlying cells (Fig 2A)

Small flat cells were occasionally found between Claudius cells and the spiral ligament Claudius cells started their degeneration already at this time These small flat cells contained many vacuoles in the dark cytoplasm and seemed to be part of the outer sulcus cells (Fig 2B) In the later stage, they seemed to become a lining on the basilar membrane spiral ligament, increasing their cytoplasmic volume and number at the site of disappearing Claudius cells

In the spiral limbus of the 4 and 6-day specimens, the cytoplasm of the interdental cells was shrunken and contained many vacuoles and lipid like materials of varying size (Fig 3A) In the 6-day specimens the horizontal parts and bodies of the degenerated interdental cells were on the verge of disappearance In this stage, the single cell membrane which seemed to derive from the limbus epithelium cells which were the epithelial cells of Reissner's membrane, turned toward the upper surface of the spiral limbus and located on it and/or epithelial cells of Reissner's membrane began to extend toward the vertical lip along the upper surface of the limbus During this process they covered the vertical orifices of flask-shaped spaces left by the disappearance of the degenerated interdental cells Much debris still remained in the vacuolated spaces (Fig 3B) Frequently, at this time, the inner sulcus cells started their degeneration and the inner sulcus had no epithelial cells

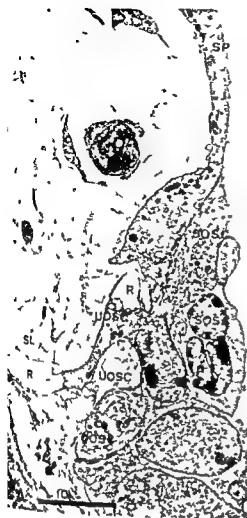
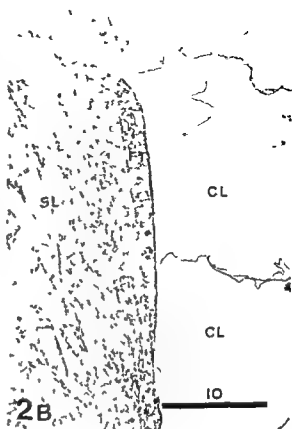


Fig 4A. Prominence cell (B) 6 days after perfusion. 20% flat cell (FT) between Claudius cells (CL) and spiral ligament (SL). Arrows in the inset indicate the corresponding area.



The spaces in the spiral ligament left by disappearance of the roots remained empty and after disappearance of the underlying cells their orifices to the endolymphatic space were closed by the cuboidal cells (Fig 4B). It was assumed that these cuboidal cells transformed from the superficial outer sulcus cells. The cuboidal cells were contiguous with the flat single cell membrane which replaced Claudius' cells on the basilar membrane and extended to the collapsed organ of Corti (Fig 5).

The spiral prominence epithelia were normal in shape but their cytoplasm were darker than normal due to increased filaments.

The tectorial membrane was detached from the degenerated interdental cells and was sometimes rolled up. There were three types of detachment of tectorial membrane from the upper surface of the spiral limbus, 1) completely free from spiral limbus and floating in the scala media (Fig 6A), 2) barely

(b) Changes in the later stage (7 to 137 days after perfusion). On the lateral wall in the 9-day specimens, although their cytoplasm is condensed and their volume reduced, superficial outer sulcus cells still remained and covered the degenerated underlying cells which were becoming vacant (Fig 4A). After the 9th day the underlying cells and roots of the outer sulcus cells completely disappeared.

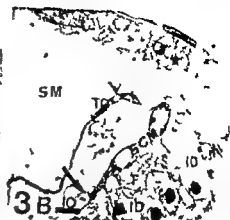
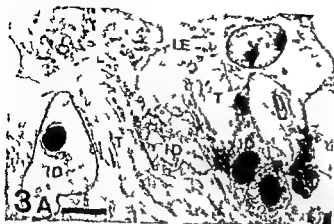


Fig 3 (A) 5A 0 0 0

to extend toward the vestibular lip from the epithelium of Reissner's membrane (*) or from the epithelium (LE) cover the vacant space after disappearance of interstitial cells (ID). Arrow shows the trace of single cell layer membrane (SCM). SM = single cell layer membrane. T = Fuschke's teeth. TC = detached tectal membrane.

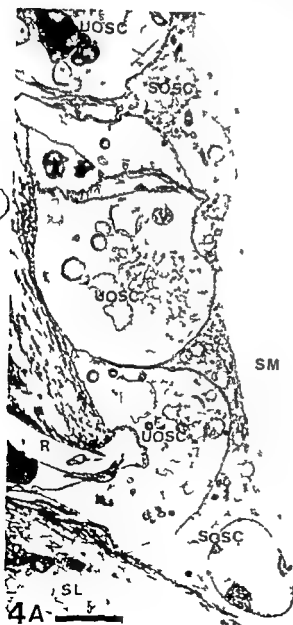


Fig 4 (A) 9 days after perfusion. UOSC = upper sulcus cells lose normal cell ganglia and become vacuolated. Superficial cells (SO) cover the degenerated underlying cells. R = vacant space after disappearance of root cells. SL = spiral ligament. 9 days after perfusion. 0 = SM = cuboidal cell endolymphatic spaces of vacuolated spaces left by disappearance of roots (R). SM = scala media.



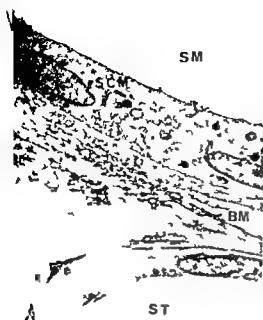


Fig 5 10 days after perfusion 20% SM Single-cell layer membrane (SCM) replace Claudius cells SM scala media ST scala tympani SL spiral ligament BM basilar membrane

the vestibular lip of spiral limbus (Fig 6B) 3) detached from spiral limbus and moved to the site of the degenerated organ of Corti (Fig 6C)

On the 8-9-day specimens the horizontal parts and the body of the interdental cell disappeared leaving flask shaped empty spaces in the spiral limbus. The single cell layer membrane covered all upper orifices of flask shaped empty spaces (Fig 7A) and then enveloped the vestibular lip in 9 day specimens (Fig 7B) and with progress of time reached the inner sulcus cells (Fig 7C). If the rolled up tectonal membrane was connected with the spiral limbus the single cell layer membrane proliferated over the tectonal membrane.

The cells of the single cell layer membrane were contiguous with the epithelial cells of the inner sulcus membrane and were morphologically quite similar to them. They were cuboidal or flat in shape and had many vesicles. Well developed Golgi complex endoplasmic

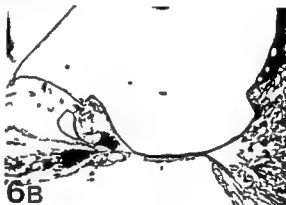
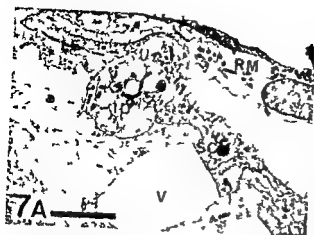


Fig 6 Fate of tectonal membrane (A) 10 days after perfusion 20% SM Completely free from the spiral

membrane



reticulum multivesicular body and mitochondria in their cytoplasm. They had many microvilli on their endolymphatic surface and had a basement membrane on the side of spiral limbus (Fig 7). The cell layer possessed junctional complexes near the endolymphatic surface and interdigitating processes. The morphological appearance of single cell layer membrane from the lateral wall of the cochlear duct (Fig 8) was the same as the above described single-cell layer membrane from the spiral limbus.

After the disappearance of the inner ear cells in the more advanced stage the cell layer membrane replaced them and tended to the collapsed organ of Corti. The single cell layer membrane finally covered the organ after merging with the single-cell layer membrane originating from the lateral side of 12–17 day specimens (Fig 8). After the appearance of the supporting cells in the organ of Corti, macrophages, fibroblasts, myelinated and unmyelinated nerve fibers and sometimes rolled up tectonic membrane were found beneath the single cell layer membrane (Fig 9).

In the 137 day specimen the degenerative process appeared to cease. Macrophages and fibrocytes were almost non-existent in the organ of Corti. Except for the stria vascularis and Reissner's membrane, the epithelial cells normally lining the cochlear duct were lost. After their disappearance the single-cell layer membrane covered the spiral ligament, the basilar membrane and the spiral lamina but did not replace the stria vascularis, although their thickness had decreased (Fig 10). In some specimens even in this stage

Fig 7 Single cell layer membrane (SCM) on spiral limbus. (A) 10 days after perfusion on 70th SM. Single cell layer membrane continues from the epithelial cell (EC) and Reissner's membrane (RM) and covered the vacuolar space (V) by disappearance of interdental cells. (B) 10 days after perfusion on 70th SM. Single cell layer membrane (SCM) developing the vacuolar space of spiral limbus (VL). (C) 10 days after perfusion on 70th SM. Reaching the inner ear cell (ISC). Arrow in the inset shows the current area in same specimen.



HA Habenulla perforata BM Basilar membrane TM rolled up tectorial membrane covered by the single-cell layer membrane Arrows show the spearhead of the single cell layer membranes

epithelial cells of Reissner's membrane at the site of the organ of Corti
D degenerated cells of organ of Corti

distorted tectorial membrane covered by single-cell layer membrane was still recognized on the basilar membrane or on the spiral limbus

(1) Group B (perfused with 2% SM solution)

In this group, the pathological changes in the epithelial cells lining the cochlear duct were restricted to the hair cells in the organ of Corti. Although not all the hair cells underwent degeneration, the defects of the reticular lamina produced by loss of outer hair cells were replaced by the hypertrophied Deiters' cells and outer pillar cells. We found no further involvement even in animals kept alive for long periods. Degeneration of the outer sulcus cells and interdental cells was not observed and no extension of the single-cell layer mem-

brane was not observed at the lateral wall and the median border of the spiral limbus. As described previously, the number of nerve fibers was reduced (Terayama et al, 1977)

(3) Group C (perfused with Ringer's solution)

In this control group, most of the organ of Corti and other epithelial cells lining the cochlear duct showed no pathological changes, even after long survival following the perfusion. A few Corti organs lost their outer hair cells, which were replaced by Deiters' cells and outer pillar cells as in group B.

DISCUSSION

It has been confirmed from tracer studies (Ilberg, 1968a, b, 1970a, b, Duvall & Suther-

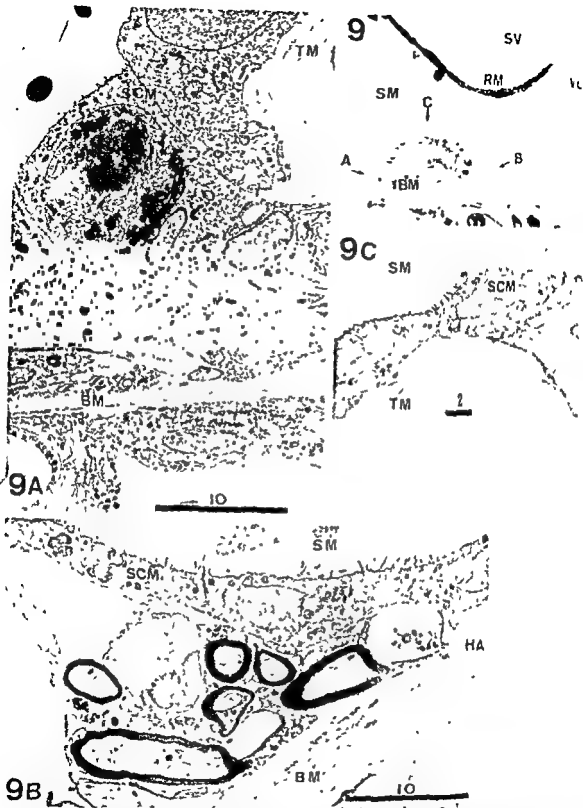


Fig 9 64 days after perfusion 20% SM Single-cell layer membrane in the area of the organ of Corti which degenerated out. Arrows in the light microscopic picture indicate the corresponding area in electron microscopic pictures. RM Reissner's membrane BM Basilar membrane VL Vestibular lip of spiral limbus SM Scala media SV Scala vestibuli (A) 9 days after perfusion 20% SM Lateral area of degenerated organ of Corti Under the single cell layer membrane, distorted tectorial membrane (TM),

unmyelinated nerve fibers and fibrocytes are found Basilar membrane (B) 64 days after perfusion 20% Median area of degenerated organ of Corti. Nerve fibers and unmyelinated nerve fibers, and fibrocytes are found BM Basilar membrane HA Habenula perforata (C) 20 days after perfusion 20% SM Top of degenerated organ of Corti Under the single-cell layer membrane, degenerated tectorial membranes (TM) are found

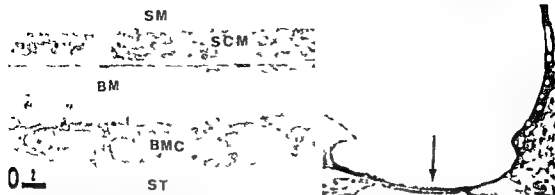


Fig. 1. 137 days after perfusion. 20% SM. Single cell layer membrane (SCM) on the basilar membrane. Arrow in the light microscopic picture indicates the corresponding area in same specimen. SM: Scala media. ST: Scala tympani. BM: Basilar membrane. BMC: Basilar membrane cell.

ing area in same specimen. SM: Scala media. ST: Scala tympani. BM: Basilar membrane. BMC: Basilar membrane cell.

id 1972, Hinojosa, 1972, Angelborg, 1974, Burke 1973, Gorgas & Jahnke, 1974) and observations on freeze fractured materials (Jahnke 1974, Iurato et al., 1976) of the cochlea that the epithelial cells and their zonulae adherentes lining the endolymphatic duct constitute the per endolymph barrier. These epithelial cells seem to have different properties regarding absorption of tracers in perilymph as pointed out by Duvall & Sutherland (1970) and Hinojosa (1972).

In our study, when the specimens were perfused with the 20% SM solution, the degeneration started at different times in different epithelial cell groups: hair cells of the organ of Corti were affected first, the interdental cells and roots of the outer sulcus cells second, and finally other epithelial cells such as 'audius' cells and the inner sulcus cells and ultimately they were replaced by the single cell layer membrane. Only stria vascularis and Reissner's membrane did not disappear and were not replaced by the single cell layer membrane. This time difference in degeneration indicates the differences in absorption property and/or the difference in vulnerability of each cell group to SM.

It was impressive that while the damage of epithelial cells lining the cochlear duct was very severe and extensively spread when perfused with 20% SM, only hair cells were damaged with 2% SM. It seems that the pro-

gressive degeneration in the cochlear duct was only expected when the threshold concentration of SM was reached in the perilymph. Ilberg et al. (1974) concluded from their investigation that signs of inner ear toxicity from aminoglycoside antibiotics appeared above their threshold value only in the inner ear fluid. Their conclusion was concerned with the toxic effects on the sensory epithelial cells only, whereas our study also showed a similar result on the extrasensory epithelium.

Although a single cell layer membrane covering the collapsed organ of Corti or "rolled up" tectonial membrane was repeatedly described in reports on the cochleae of animals and humans of viral infection (Lindsay et al., 1960, Nager, 1952), acoustic trauma (Ward & Duvall, 1971), sudden deafness (Beal et al., 1967), Kanamycin administration (Benitez et al., 1962) and mechanical disruption of the organ of Corti (Duvall et al., 1969), they all failed to describe the origin of the single cell layer membrane. We found in this study that it originated from the epithelial cells of Reissner's membrane and/or limbus epithelium cells medially, and from the superficial outer sulcus cells laterally. These two single cell layer membranes which originated from median and lateral sides merged on the site previously occupied by the organ of Corti before its degeneration.

The repair reaction of epithelial cells of

Reissner's membrane after its traumatic rupture is well known (Laurence & Yantis, 1957, Duvall & Rhodes, 1967). We found a similar strong repair process in the single cell layer membrane. There is a morphological resemblance between the epithelial cell layer of Reissner's membrane and the single-cell layer membrane, but its physiological significance is not clear. Assumedly, the membrane develops in order to block communication between perilymph and endolymph.

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ZUSAMMENFASSUNG

Ultrastrukturelle Veränderungen des extrasensorischen Epithels in der Scala media der Meerschweinchen Cochleae wurden 3 bis 137 Tage nach einer perilymphatischen Perfusion der Schnecke mit 20% Streptomycin beobachtet. Die Degeneration begann im Cortischen Organ schrittweise zu den Interdentalzellen und den Wurzeln der äußeren Sulcuszellen fort und umfaßte schließlich andere Epithelzellen wie Claudiuszellen und die inneren und äußeren Sulcuszellen. Im Schlußstadium ausgeschlossen die Stria vascularis und die Reißnersche Membran wurden alle Epithelzellen die die Ductus Cochleae linieren mit einer einschichtigen Zellmembran die zentral von den Epithelzellen der Reißnerschen Membran und seitlich von oberflächlichen äußeren Sulcuszellen hervorgingen ersetzt.

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A TEST BATTERY APPROACH TO THE INVESTIGATION OF SUSCEPTIBILITY TO TEMPORARY THRESHOLD SHIFT

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Abstract This study examined individual differences among various psychophysical measures that have been used previously as predictors of susceptibility to induced temporary threshold shift (TTS). The test battery was administered to a group of five normal hearing young adults and consisted of the following procedures:

(1) critical intensity (CI) at 2 kHz, (2) loudness

level at 2 kHz, and two others using tonal fatiguers were utilized to assess susceptibility to TTS. Results revealed that the test battery delineated tough from tender ears. Furthermore, the aural overload test was found to be a highly accurate predictor of TTS.

Individual differences in susceptibility to noise trauma are well recognized. For obvious reasons, investigators have long attempted to develop a measure which might delineate those persons most susceptible to hearing loss from high noise levels. Much of this research has focused on the temporary threshold shift (TTS) paradigm. The rationale for using TTS was based on a hypothesis proffered by Temkin in 1933 (Ward 1973). Temkin suggested that the measurement of the temporary change in hearing sensitivity following a brief and moderately intense acoustic overstimulation provided a simple and valid estimate of eventual permanent threshold shift (PTS) incurred from more severe exposures to loud sound. Despite considerable research efforts, however, the relationship between TTS and PTS remains far from simple. Known to complicate this relationship are the several variables

which influence the amount of TTS an individual incurs. For example, the frequency, duration, and intensity of both the exposure stimulus and the test stimulus effectively alter the subsequent shift in threshold sensitivity.

Consequently, utilization of the TTS paradigm as a single simple test of general susceptibility has never been realized despite numerous attempts to do so. Ward (1965, 1968) for instance, concluded that the best predictor of eventual PTS was the TTS that resulted from exposure to *that specific noise* to which the individual is routinely exposed in industry. This very same feature of noise specificity associated with the TTS paradigm, on the other hand, limits the clinical utility of TTS methods in hearing conservation programs. To explain Ward's conclusion suggests that a single standard noise stimulus can not be administered to all subjects. Rather, the noise from the individual's specific work environment must be employed. Hence, the use of the TTS paradigm as a test of susceptibility has recognized limitations.

In search of alternatives and/or additions to the conventional TTS procedure, this study was designed to examine individual differences among various psychophysical measures that have been suggested previously as potential predictors of TTS and auditory fatigue. More specifically, three tests, one of which incorporated a TTS strategy, were examined. First, is the aural overload test, originally proposed by Lawrence and Blanchard (1954) as a

measure of susceptibility and later used in differential diagnosis (Lawrence & Yantis, 1956). Loudness discomfort level (LDL) was another measure used in this investigation. Hood (1968) has suggested that LDL may be related to the amount of post-stimulatory fatigue an individual incurs. The final measure, referred to as the critical intensity (CI) procedure, was conceived originally by Ruedi (1954) and later used by Ward (1965, 1968). In the traditional CI paradigm the subject is exposed to a fatiguing stimulus which is increased successively in level until a criterion amount of TTS is observed. In the former investigations by Ruedi (1954) and Ward (1965, 1968) the only time intervening between successive exposures was that required for threshold determination. That is, no recovery period was employed. A modification of the CI concept was employed in this study. Our procedure seeks to determine the CI at which maximum TTS shifts upward in frequency from the exposure frequency to one half octave above the exposure frequency, a feature which appears to distinguish adaptation processes from fatigue (Ward, 1973). The present CI procedure was modified further from the earlier techniques by allowing recovery periods between exposure levels.

To assess susceptibility to TTS, three exposure stimulus/test stimulus combinations were used. The first combination utilized broad band noise as the fatiguer and measured threshold at 4 kHz following exposure. This combination was selected for two primary reasons. First, there has been substantial research conducted in which a broad band noise/4 kHz combination was used (Lightfoot, 1955; Miller, 1958; Ward, Glorig & Sklar, 1958; Humes, Schwartz & Bess, in press). Secondly, a broad band fatiguer has been recommended as the most appropriate standard TTS index of susceptibility (Ward, 1965, 1968). In addition to the noise exposure condition, tonal overstimulation utilizing both high and low frequency fatiguing stimuli was employed.

METHOD

Subjects

Five normal hearing adults (1 male and 4 females) ranging in age from 23 to 24 with a mean age of 23.3 years comprised the subjects of this study. All subjects exhibited normal tympanometric curves (normal amplitude shape, middle ear pressure within ± 70 mmH₂O), normal acoustic reflex thresholds (less than 95 dB HTL at 0.5 through 4 kHz in the test ear), and pure tone air-conduction thresholds of 5 dB HTL (ANSI 1969) for the frequencies 0.5, 1, 2 and 4 kHz.

Apparatus

Pure tone air conduction screening of all subjects was accomplished with a clinical diagnostic audiometer (Grason Stadler Model 1701). Electroacoustic impedance measurements were obtained with an impedance bridge (American Electromedics E-83).

The fundamental and exploring tones for the aural overload test were supplied by two oscillators (General Radio Model 1309A and Tektronix Model SC502) and monitored both a frequency counter (Tektronix Model DC501) and an oscilloscope (Tektronix Model 5103N). The output of the attenuator was to an electrodynamic earphone (Telephonics TDH-39, 10 ohm mounted in an MX-41 cushion).

In the assessment of LDL, the test stimulus was supplied by an oscillator (General Radio Model 1309A), monitored by a frequency counter (Tektronix Model DC 501) and by a timer (Grason Stadler Model 1223) and an electronic switch (Grason Stadler Model 1287B) which maintained a pulse duration with an on time of 300 msec, an off time of 700 msec and a rise/decay time of 10 msec. The tone was then fed to an amplifier (Wentworth Model 465A) and recorded through the attenuator and finally to the same earphone that was used in the aural overload test.

The systems used for the aural overload and loudness discomfort procedures included a

phones, were periodically checked for harmonic distortion. For the signals generated by the oscillator, the levels of the 2nd, 3rd, and 4th harmonics at the earphone, were at least 20 dB below that of the fundamental tone. In addition, the linearity of the recording attenuator was confirmed over the range of use (65 dB). The clinical audiometer, employed in this investigation for screening, CI, and determination of TTS, was calibrated in accordance with ANSI S3.6 (1969) specifications. In addition, the overall sound pressure level (SPL) and the spectrum shape of the white noise stimulus used in the TTS studies were determined.

Calibration checks were performed throughout the investigation and upon completion of the study. All testing was completed in double-blind testing suites (IAC Series 1200) having an acoustic environment suitable for threshold determination (ANSI, 1960).

Procedure

The fatiguing nature of some portions of the present investigation required that the listening task be divided into three separate experimental sessions. During the initial session, pure tone air-conduction thresholds were obtained in the test ear at octave intervals ranging from 0.5 to 4 kHz. Electroacoustic impedance measurements were also made to establish normalcy of middle ear function. In addition to the screening, one of the following test categories was randomly selected for completion during the initial session with the remaining categories assigned to later sessions:

- Loudness discomfort level measured in accordance with Morgan et al. (1974) at frequencies 0.5, 1, 2 and 4 kHz.
- TTS at 0.75 and 3 kHz recorded from 0 to 3 minutes post exposure following 3-minute exposures to 0.5 and 2 kHz pure tones at 100 dB SPL.
- Aural overload thresholds as measured according to Faust (1971) for the fundamental frequencies of 0.5, 1 and 2 kHz.

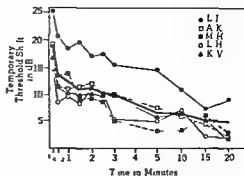


Fig. 1. TTS at 4 kHz as a function of recovery time for the 5 subjects of this study. Heavy line represents mean data for the group.

(See Humes, 1977 for details of Faust's technique.)

(b) TTS at 4 kHz recorded from 0 to 20 minutes post exposure following stimulation with thermal noise at 110 dB SPL for 5 minutes.

3. CI procedure at 2 kHz

Sweep frequency threshold tracings from 2–4 kHz were obtained after exposure to a 2 kHz pure tone for one minute at 90, 95, 100, 105 and 110 dB SPL. Ten minute rest intervals followed each exposure level.

The procedural groupings were presented in a random fashion to avoid possible stimulus order effects. Each session was followed by at least 24 hours of recovery after which pure tone thresholds at octave intervals from 0.5 through 4 kHz were redetermined. If thresholds had not returned to resting level (± 2 dB), another 24 hours intervened. In addition, subjects were instructed to avoid any noise exposure between test sessions.

RESULTS

Temporary Threshold Shift (TTS)

The TTS obtained in the normal hearers at 4 kHz following exposure to 110 dB SPL broad band noise for five minutes is illustrated in Fig. 1. The mean data are indicated by the heavy

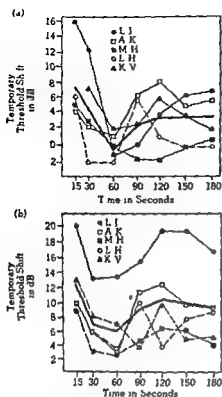


Fig 2 Individual variability in TTS as a function of recovery time. Heavy line represents mean data for group. (a) TTS at 0.75 kHz following exposure to 0.5 kHz pure tone at 100 dB SPL for 3 min. (b) TTS at 3 kHz after stimulation with 2 kHz tone at 100 dB SPL for 3 min.

solid line while individual data are marked according to the key of this figure. Examination of the TTS at 2-minutes post exposure (TTS_2) was compared with predicted TTS derived from the formula developed by Ward, Glong, and Sklar (1958). The predicted TTS_2 for the exposure conditions utilized in this investigation is approximately 12 dB. As seen in Fig 1, subject L.J. demonstrated threshold shifts that far exceeded both predicted values and those of the other subjects. Concurrently, subject M.H. demonstrated resistance to TTS. As a group, however, the mean results of the present study are in excellent agreement with those predicted according to the equation of Ward et al. (1958).

Figure 2 illustrates the TTS recorded at 0.75 and 3 kHz following three minute 100 dB SPL exposures to 0.5- and 2 kHz pure tones, respectively. The heavy line in Fig 2 (a & b)

provides the mean data for the subjects. Consistent with previous research (Hirsh & Ward 1952, Hirsh & Bilger, 1955) TTS values were extremely variable across subjects. In agreement with prior investigations, the appearance of a "bounce" in the short-term recovery pattern (Hirsh & Ward 1952, Singer & Tillman, 1970). Using the mean as an arbitrary limit, one subject (L.J.) consistently exceeded this limit and frequently yielded the highest value of TTS. In addition, as shown in the previous figure, subject M.H. exhibited the greatest resistance to TTS. Thus, for these two subjects there appears to be a general condition of susceptibility or resistance to TTS, independent of the frequency examined.

In view of the consistency of the T-rankings observed thus far, these data were analyzed further via the Pearson r correlation index (Downey & Heath 1974). A high positive correlation was obtained between noise-induced TTS at 15 seconds post exposure ($TTS_{0.25}$) for the 4 kHz test tone and the tone-induced $TTS_{0.25}$ recorded at 0.75 kHz ($r=0.86, p<0.05$) and 3 kHz ($r=0.80, p<0.05$). For the TTS_2 values, a significant correlation was obtained for the 4 kHz and 3 kHz test tones only ($r=0.80, p<0.05$).

Critical Intensity

The results obtained from the critical intensity procedure are illustrated in Fig 3. The scissa represents the difference in TTS at

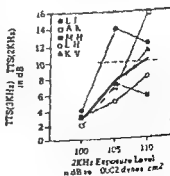


Fig 3 Mean (heavy line) and individual results plotted with the critical intensity (CI) procedure.

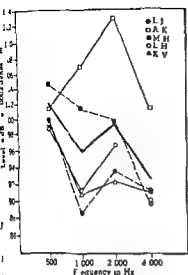


Fig. 4. Mean (heavy line) and individual loudness discomfort levels (LDL) for the 5 subjects of this investigation.

exposure frequency (2 kHz) and TTS at the half octave above this frequency (3 kHz). Data for the 90 and 95 dB exposures are not provided because a clear pattern failed to develop at these intensities. The remaining exposure intensities, however, yielded an interpretable pattern. At the 105 dB exposure intensity one subject (L J) exceeded an arbitrarily defined cut off value of 10 dB (same criterion as that used by Ward 1968). Following the 110 dB exposure, subjects A K and L J demonstrated the greatest upward shift in TTS while subject M H displayed the least amount. Overall, however, the CI levels from this study are compatible with those obtained by Ward (1968) using a similar procedure.

Loudness Discomfort Level

Results of the LDL data are illustrated in Fig. 4. With the exception of subject A K, individual data demonstrated a common pattern of frequency dependence. The frequency dependence of the LDL estimates obtained in this study is comparable to that reported by Morgan et al. (1974) although the present results are approximately 20 dB lower. Subject L J is seen to display a generally lower mean

LDL (average LDL for 0.5 through 4 kHz) while subject M H demonstrated a relatively higher mean discomfort level. This ranking, therefore, is in reasonable agreement with the TTS ranking discussed above.

Loudness discomfort levels, however, were found to be poorly correlated to TTS with coefficients varying between 0.26 and -0.17. It is important to note, however, that the subjects of the present study received no training with the experimental task prior to the determination of LDL. On the other hand, it is desirable that a potential measure of susceptibility be as free from training restrictions as possible to facilitate broad and rapid application. This apparent pitfall of the LDL procedure is discussed in greater detail below.

Aural Overload

Thresholds obtained with the modified aural overload test are provided in Fig. 5. Again with the exception of the 0.5 kHz fundamental tone, subjects L J and M H are at the extremes of the data continuum. In addition, subject L J is clearly delineated from the group at frequencies 0.5 and 1 kHz. When the group data were subjected to statistical anal-

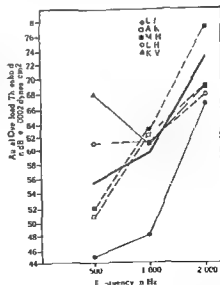


Fig. 5. Mean (heavy line) and individual aural overload thresholds for fundamental frequencies 0.5, 1 and 2 kHz.

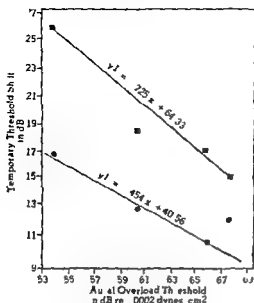


Fig. 6. Prediction of $TTS_{0.5}$ (S.E. = 1.05 dB) and TTS_2 (S.E. = 1.92 dB) at 4 kHz from the mean aural overload threshold (0.5, 1 and 2 kHz).

ysis aural overload thresholds were found to be significantly ($p < 0.05$) correlated to TTS. The aural harmonic threshold at 2 kHz was negatively correlated to $TTS_{0.5}$ ($r = -0.87$) and TTS_2 ($r = -0.89$) for the 3 kHz test tone. Furthermore, the mean aural overload threshold (average overload threshold for 0.5, 1 and 2 kHz) was highly correlated to $TTS_{0.5}$ and TTS_2 at 4 kHz. The correlation between mean overload and $TTS_{0.5}$ at 4 kHz was -0.98 while the correlation between mean overload and TTS_2 at 4 kHz was -0.84 . Hence the lower the aural harmonic threshold the greater the TTS. This finding is consistent with previous physiological hypotheses (Lawrence & Blanchard 1954; Drescher & Eldredge 1974).

In view of the relationship between mean aural overload and TTS at 4 kHz following exposure to thermal noise, linear regression equations were calculated (Fig. 6). The amount of $TTS_{0.5}$ at 4 kHz following exposure to broad band noise of 110 dB SPL for 5 minutes can be predicted from the mean overload threshold by utilizing the following equation: $y = -0.725x + 64.33$ where y is equal to

predicted $TTS_{0.5}$ in dB and x is equal to 1 mean aural overload threshold. TTS_2 can be predicted from mean overload (x) with the following formula: $y = -0.454x + 40.56$. The standard error of the regression lines was 1.05 dB for the prediction of $TTS_{0.5}$ and 1.92 dB for the prediction of TTS_2 .

DISCUSSION

The statistically significant positive correlations among tone induced and noise induced $TTS_{0.5}$ at first appear to be in opposition to results obtained by Ward (1965, 1968). Ward found that those persons most susceptible experimentally induced threshold shifts induced by high frequency stimuli were not necessarily the most susceptible to low frequency overstimulation. That is, a pattern of general susceptibility did not appear to exist in his subjects. The correlation of $TTS_{0.5}$ in this study for low and high frequency stimulation however does not stand in opposition to Ward's conclusion. Ward (1968) was concerned primarily with measures of fatigue ($TTS_{0.5}$ in this study) rather than adaptation ($TTS_{0.25}$). Adaptation has proven to be fairly constant as a function of frequency (Hood 1950) and should therefore be correlated across frequency. Moreover, failure to achieve a statistically significant correlation between $TTS_{0.5}$ at 0.5 kHz and high frequency TTS_2 (3 and 4 kHz) is in accord with previous observations concerning auditory fatigue (Ward 1965, 1968). It should be noted however that the adequacy with which low frequency TTS reflects underlying cochlear alterations has been questioned recently (Humes in press). The relative insensitivity of low frequency TTS may therefore be responsible for the lack of correlation between low and high frequency TTS observed previously by Ward and in the present study. Similar factors may govern the correlation between aural overload thresholds and T

all that significant correlations observed in present study were restricted to high frequency TTS. Humes & Schwartz (1977) commented on this frequency dependent relation. Briefly they suggested that low-frequency pure tone thresholds are less sensitive to apical cochlear lesions than comparable overload thresholds. The relative insensitivity of low frequency pure tone thresholds to cochlear status has been noted previously by several investigators (Crowe, Guild & Polvogt, 1968; Bredberg 1968; Bohne, Eldredge, and Henderson, 1973; Hamernik and Sitler, 1975a, 1975b, 1975c; Corso, 1976; Suga & Lindsay, 1976). Hence, a lack of relation between low frequency overload thresholds and low frequency cochlear damage measured via pure tone thresholds may merely reflect the insensitivity of the latter to alterations in the apical portion of the cochlea.

It has been suggested that a brief (3-5 min) monaural exposure to broad band noise, such as that used in this study, may represent the single most effective predictor of general susceptibility to TTS aside from the specific dose to which the individual is exposed (Eldredge 1965, 1968). The high correlations observed between mean overload threshold and PTS induced by such a broad band noise, therefore, argues for the validity of aural overload thresholds as predictors of overall susceptibility to TTS. Whether thresholds of overload prove to be accurate predictors of permanent threshold shift awaits further investigation. Physiological data directed to this issue suggest that such a relation may prove true (Eldredge & Eldredge 1974). As indicated in the introduction, however, the TTS-PTS relationship is not a simple one. Hence, the relation of aural overload thresholds to PTS requires direct confirmation.

The present investigation represents an initial step in developing a possible battery of tests used to delineate susceptible from non-susceptible individuals. The findings of this study suggest that both the aural overload test

and the CI procedure possess sufficient sensitivity to underlying individual differences in TTS. Furthermore, as indicated by the present results, the effectiveness and applicability of the latter could be enhanced by restricting exposure levels to 100, 105, and 110 dB SPL and excluding the lower exposure intensities (90 and 95 dB SPL). This would also reduce the time of test administration considerably.

It would appear, on the other hand, that loudness discomfort level is an inappropriate measure of susceptibility to TTS. Not only were poor correlations of LDL to TTS established in this study, but the determination of LDL itself is largely a function of instructions given to the subject, the psychophysical method used, and the amount of subject training (Davis et al., 1946; Silverman 1947; Morgan, Wilson & Dirks 1974; Humes, 1976). It is the latter factor which possibly poses the most serious problem to the use of LDL as a measure of susceptibility. The need for subject training requires more time than is often available in a screening program. This is especially true if the test in question is one of several tests comprising a battery of measures.

There are other measures of susceptibility that do not incorporate a TTS methodology and were not investigated here. The threshold of octave masking (TOM) test (Clack & Bess 1969), for instance, appears to be sensitive to individual differences in noise induced TTS (Humes 1977; Humes, Schwartz & Bess, in press). Another proposed index of susceptibility that has received relatively little attention is word discrimination score obtained in noise (Humes 1977; Humes, in press). Thus the present findings, when considered with other recent research, suggest that the following procedures would comprise an ideal susceptibility test battery: (1) the aural overload test, (2) the TOM test, (3) the CI procedure (appropriately modified) and possibly (4) word discrimination scores obtained in noise. The relative advantages and disadvantages of most of these tests has been discussed elsewhere (Humes 1977).

ZUSAMMENFASSUNG

Diese Arbeit untersucht die zeitweise Schwellenverschiebung (TTS) bei verschiedenen Frequenzen und Intensitäten. Die Versuchsschritte bestanden aus einer Gruppe von fünf normalen Hörern. Das Experiment bestand aus folgenden Stufen: 1) Kritische Intensität (CI) zu 2 kHz; 2) Lautheitsunbehagengrad (LDL) zu 0,5, 1, 2 und 4 kHz und 3) Ohrenüberbelastungsschwellen zu 0,5, 1 und 2 kHz. Die Ergebnisse zeigten, daß die Versuchsschritte Widerstandsfähigkeit von empfindlichen Ohren unterschieden hat. Weiterhin zeigte sich, daß der Ohrenüberbelastungstest die zeitweise Schwellenverschiebung außerordentlich genau vorhersagen kann.

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COMBINED EFFECTS OF NOISE AND NEOMYCIN

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Abstract Cochlear damage resulting from the combination of neomycin with acoustic overstimulation was investigated in guinea pigs. Four groups of animals received subcutaneous injections and exposure to broad band noise daily for 7 days as follows: I. Neomycin (200 mg/kg) followed by 10 hours of noise at 115 dB SPL. II. Saline followed by 115 dB noise. III. Neomycin followed by low intensity noise (45 dB as an acoustic control) or IV. Saline followed by 45 dB noise. After a 30 day stabilization period, each ear was examined electrophysiologically and histologically. Measures of cochlear integrity included AC cochlear potentials from 100 Hz through 30 kHz as well as outer hair cell (OHC) counts. A marked interaction leading to augmentation of damage was found when neomycin was combined with 115 dB noise (Group I). Losses in cochlear sensitivity averaged across all frequencies amounted to 62 dB in Group I whereas the averaged losses for Groups II and III were only 16 dB and 17 dB respectively. Loss of OHC's was close to 100% in Group I while OHC losses were only 17% in Group II and 26% in Group III.

The ototoxic effects of the aminoglycoside antibiotics include damage to hair cells of the organ of Corti as well as changes in electrophysiological measures of cochlear function (Hawkins, 1959; Falk, 1972; Brummett, 1973; Ylikoski, 1974). In general, damage occurs first in outer hair cells at the base of the cochlea. With continued administration of the drug, outer hair cells toward the apex as well as inner hair cells may become involved (Ward & Fernandez, 1961; Benitez-Schuknecht & Brandenburg, 1962; Reddy & Igarashi, 1962; Hawkins & Engstrom, 1964).

Damage as a result of severe acoustic exposure is similar in many respects to that pro-

duced by the aminoglycoside antibiotic. Whether the exposure consists of a pure tone or a narrow band of noise. Again outer hair cells appear more vulnerable than inner hair cells (Lune, Davis & Hawkins, 1944; Berg, 1965; Lim & Melnick, 1971). However, the extent the location of damage within the cochlea is determined by the frequency spectrum of the acoustic exposure (Stockwell, Axel, Engstrom, 1969; Fried, Dudek & Bohne, 1971; Spoendlin, 1976).

Because of the widespread use of the aminoglycoside antibiotics and the presence of high levels of noise in hospital and environmental areas, there are significant possibilities for interaction between these two agents. To date, however, there has been relatively little experimental investigation of such possibilities (Dayal, Koshanian & Mitchell, 1971; Järta, Järta, Kohonen & Järta, 1972; Dayal, Barek, 1975; Hawkins, Marques & Clark, 1975; Marques, Clark & Hawkins, 1975). Furthermore, the results from these investigations are not in complete agreement with each other.

The aminoglycoside antibiotic kanamycin has received the greatest amount of attention in regard to the possibility of an interaction with noise. Histological examination of the cochlea in guinea pigs exposed to both kanamycin and noise, was first reported to be a potentiation of effect by Dayal et al. (1971). This work involved low level noise (45 dB SPL).

Table 1

Group	Acoustic exposure level*	Drug treatment
I. Combination	115 dB	Neomycin sulfate (200 mg/kg)
II. Noise alone	115 dB	Normal saline
III. Neomycin alone	45 dB	Neomycin sulfate (200 mg/kg)
IV. Procedure control	45 dB	Normal saline

* All sound pressure levels (SPLs) in this report are referred to 0.0002 μ bar

and low doses of kanamycin (15–50 mg/kg) potentiation of cochlear damage was also reported in work by Dayal & Barek (1975) at higher doses of kanamycin (100 mg/kg) and noise levels of 90 dB. The duration of treatment in these studies was on the order of 4–5 weeks.

More recent work has not confirmed the findings described above. Hawkins et al (1975) and Marques et al (1975) have reported extremely variable findings after treating guinea pigs with various combinations of kanamycin and noise. In these investigations both electrophysiological and histological measures were used, interaction between noise and drug was seen only in certain animals and only with noise exposures of at least 100 dB. These investigations involved treatment durations of 7 days.

The only other aminoglycoside antibiotic which has been studied in combination with noise is neomycin. Augmentation of damage by combination of noise with neomycin was reported by Jauhainen et al (1972), following daily exposure of guinea pigs to 115 dB of octave band noise combined with daily injections of neomycin (200 mg/kg). The duration of treatment was 7 days. Both electrophysiological and histological measures were reported to demonstrate the augmentation effect.

The above results with noise and neomycin have not been replicated prior to the present report. Because of the conflicting evidence concerning the possible interaction between

the aminoglycoside antibiotics and noise, the present investigation was designed to determine whether in fact the observations concerning neomycin and noise could be confirmed. In addition, although the study by Jauhainen et al appears to have been carefully done, their electrophysiological measures did not extend to frequencies higher than 4 kHz. In view of the well known tendency for ototoxic drugs to preferentially damage the basal portion of the cochlea the present investigation extended the observations to include frequencies up to 20 kHz.

MATERIALS AND METHODS

Thirty-two healthy pigmented guinea pigs weighing between 200–400 g were selected for this study. The animals were randomly assigned to one of four groups of 8 to receive the treatments as specified in Table 1.

The acoustic exposure was a broad band of white noise (Fig. 1). The 115 dB exposure level was chosen to be consistent with the earlier work of Jauhainen et al (1972). The low level exposure (45 dB) was added to provide an innocuous sound environment as an acoustic control.

Exposure to noise was maintained for 10 hours/day over 7 consecutive days for a total exposure time of 70 hours. During the sound exposure the animals were confined in groups of 16 within a 0.64 cm wire mesh cage (137 cm \times 46 cm \times 30 cm) suspended in a double walled, sound shielded room. Water was available at all times. Following each day's exposure the animals were returned to their home cages where food was also available.

Noise was produced by a Lansing speaker (no. 2483) driven by a random noise generator (General Radio no. 1382) in conjunction with a filter (Bruel and Kjaer no. 2112) and a Dynaco 60 Watt power amplifier. The speaker was suspended 153 cm above the cage floor and was fitted with an exponential horn. The open end terminated 76 cm above the floor.

Sound pressure levels were measured with a sound level meter (Brüel and Kjær no 2203) on the linear setting. Measurements were made at 2 inch intervals over the entire cage floor at heights of 1, 3, and 5 inches (624 points). The sound pressure level averaged over these points did not vary from 115 dB by more than ± 2 dB.

Injections of neomycin (200 mg/kg) or of normal saline (drug vehicle in equivalent volume per body weight) were given subcutaneously immediately before each sound exposure period. All injections were given using a blind procedure.

Following the 7-day period of injections and acoustic exposure, an interval of 30–40 days was allowed for damage effects to stabilize. During this time the animals were kept in their home cages with no further exposure to drugs or to noise other than the ambient noise level within the laboratory.

At the end of the stabilization period each animal was anesthetized by an intraperitoneal injection of allobarbitol (DIAL, 60 mg/kg) with urethane (240 mg/kg). A tracheal cannula was inserted and the animals were maintained on artificial respiration. Body temperature was maintained at $38^{\circ} \pm 2^{\circ}\text{C}$.

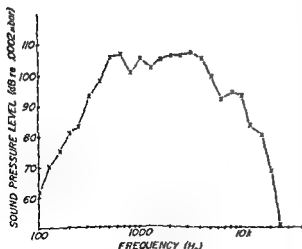


Fig. 1. Octave sound pressure spectrum of the broad band of white noise used as acoustic exposure measured at 115 dB SPL. Data points are at the center frequencies of each $\frac{1}{3}$ octave.

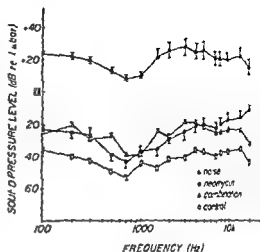


Fig. 2. Averaged $1 \mu\text{V}$ isopotential functions of the AC cochlear potential obtained from all ears of four groups of animals. Vertical range bars represent ± 1 standard error of the mean. Major losses are seen in the group which received loud noise in combination with neomycin (top of figure). Exposure to noise alone or neomycin alone resulted in much less damage. Control group is seen at bottom of figure.

Both ears of each animal were prepared for the recording of AC cochlear potentials from the round window membrane using a silver electrode with a round ball tip. For presentation of the acoustic test stimuli a specially designed sound cannula containing a calibrated probe tube microphone (Vernon & Meikle, 1974) was sealed into the external auditory meatus. The sound cannula was connected to a loudspeaker (General Radio no 555) by 25 cm of soft rubber tubing and 31 cm of rigid walled tubing.

Acoustic test stimuli consisted of pure tones at 16 frequencies from 100 Hz through 20 kHz (see Fig. 2). Stimulus level was measured at each frequency in each ear. The AC cochlear potentials elicited by these stimuli were amplified 1000 times and then measured using a narrow band wave analyzer (General Radio no 1900-A). The electrophysiological measures included (1) a measure of the amount of sound required to produce $1 \mu\text{V}$ of AC cochlear potential (the $1 \mu\text{V}$ isopotential function) at the above frequencies and (2) a measure of the magnitude of the AC cochlear potential.

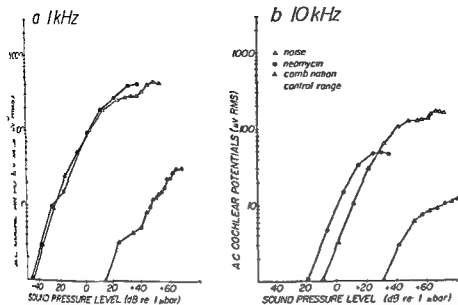


Fig. 3 Intensity functions of the AC cochlear potential (a) 1 kHz and (b) at 10 kHz. Each curve represents

the median ear from one of the three experimental groups. Stippled area shows the complete range of control values.

resulting from a sound stimulus of increasing intensity (intensity functions) at 1 kHz and 10 kHz. Electrophysiological measures are reported in terms of RMS voltage. Tests were periodically made for radiation artifact (Verdon & Meikle 1974).

Immediately following the last electrophysiological measure, the cochleas were perfused with Dalton's solution containing 1% OsO_4 as a fixative. The ears were further prepared for microscopic examination using the surface preparation technique (Engstrom, Ades & Andersson, 1966). The organ of Corti was dissected free from the osseous spiral lamina and sections were mounted in glycerine. Counts were made of missing outer hair cells (OHC's) in segments of 80 OHC lengths at the base of the cochlea, turn 2, turn 3, turn 4 and at the apex. Inner hair cells were not counted but any damage to these cells was and recorded.

RESULTS

A marked interaction between noise and neomycin was confirmed by both the electro-

physiological and histological measures. The combination of these agents was far more traumatic to the ear than either agent given alone.

Electrophysiological measures

The mean $1 \mu\text{V}$ isopotential curve for each of the treatment groups is shown in Fig. 2. In agreement with earlier work (Jauhainen et al., 1972), damage resulting from the combination of noise with neomycin was extreme. The average dB loss for the Combination group (averaged across all frequencies, relative to Control group levels) amounted to 62 dB, while for the groups receiving noise alone and neomycin alone the average losses were only 16 dB and 17 dB respectively (Fig. 2).

A similar effect was demonstrated by the intensity functions at 1 kHz and 10 kHz (Fig. 3). The groups receiving noise alone or neomycin alone were close to or within the range of the control values, while the Combination group was severely depressed relative to the Controls. Intensity function curves for the latter group were, in general, far to the right on the abscissa, indicating dramatic losses

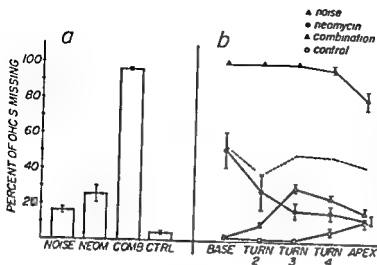


Fig 4 Results of outer hair cell counts expressed as a percentage of the total number that would exist in an equivalent segment of undamaged organ of Corti. Counts averaged over all areas of the cochlea are shown for each group of animals in (a); these counts are represented as a function of place in the cochlea in (b). Vertical bars represent ± 1 SEM. The dashed line represents the sum of effects below it at each point.

in sensitivity, and substantially depressed in height, indicating losses in the output capability of the ear (Table II, which shows the maximum output for each group as averaged values, indicates the augmentation effect clearly).

Noise alone and neomycin alone exerted approximately equivalent effects over most of the frequency range (Figs 2, 3a). At frequencies above 5 kHz, however, neomycin alone led to slightly more damage than did noise alone (Figs 2, 3b). The same comparisons are evident in Table II.

In addition, it is interesting to note that variability within the two groups receiving neomycin was considerably greater than in the Noise alone or the Control group. In these latter two groups variability was extremely small.

Table II Maximum output of the AC cochlear potential at 1 kHz and 10 kHz. Values are the means of all ears within each treatment group.

Group	1 kHz	10 kHz
Noise alone	404 μ V	185 μ V
Neomycin alone	377 μ V	51 μ V
Combination	24 μ V	9 μ V
Control	1264 μ V	318 μ V

Histological measures

Total OHC losses for each group expressed as percentage values relative to the total number of OHCs that would exist in an equivalent segment of undamaged organ of Corti are shown in Fig 4a. These OHC losses are depicted as a function of cochlear turn location in Fig 4b. We can see the dramatic exacerbation of damage effects in both Fig 4a and Fig 4b. Not represented in the figure are the inner hair cell losses which were very small in the single agent groups but nearly 100% in the Combination group.

Within every cochlear turn the number of missing OHCs in the Combination group exceeded the number missing in the Noise-alone, Neomycin alone, and Control groups combined. This comparison is indicated by the dashed line (Fig 4b) which represents the arithmetic sum of the damage effects below it at each point. Again variability was greater within the Combination and Neomycin alone groups than within the Control or Noise-alone groups.

DISCUSSION

There appears to be an interaction between noise and neomycin as shown by the Combination group. That is, the magnitude of the

losses when noise was combined with neomycin was far greater than the simple addition of losses due to noise alone and neomycin alone.

In general, the present results confirm the observations of Jauhainen et al. (1972). Some differences, however, do exist. While this earlier report showed noise to have produced consistently more damage than neomycin at frequencies up to 4 kHz, in the present study these two agents exerted approximately equivalent effects over most of the frequency range. At frequencies above 5 kHz we found damage due to the neomycin alone was greater than that due to noise alone. This may be seen in the isopotential functions as well as in the comparison of intensity functions at 1 kHz and at 10 kHz. In the earlier study, electrophysiological observations did not extend above 4 kHz which prevents a direct comparison of data above that frequency.

There was a good correspondence between the electrophysiological and histological results of the various treatments. The nearly total destruction of OHC's in the Combination group was consistent with the dramatic depression of the isopotential and intensity functions in this group. Moderate electrophysiological depression in the Noise alone and Neomycin alone groups was associated with relatively modest OHC losses in most areas of the cochlea. Neomycin alone, however, led to a more dramatic destruction of OHC's in the basal portions of the cochlea. This finding is consistent with the greater depression of cochlear potentials at high frequencies due to this agent. Additionally, neomycin treatment appears to have led to more variable results than did noise treatment as seen both electrophysiologically and histologically.

The fact that neomycin and noise, when given alone, tended to concentrate their effects in different parts of the cochlea (neomycin in the base, noise in Turn 3) might have led to the suggestion that the interaction occurring in the Combination group resulted simply from the compounding of drug induced

damage in the basal turn with noise induced damage in Turn 3. This cannot be the case, however, for Fig. 3 clearly shows that damage in both Turn 3 and the basal turn, in the Combination group, far exceeded that due to either agent alone in those same locations.

The mechanisms of the interaction between noise and neomycin remain to be elucidated. Neomycin has been shown to interfere with the turnover of phosphoinositide lipids of the organ of Corti and stria vascularis (Schacht, 1976). These lipids are thought to be involved in the control of membrane permeability. It was suggested in that report that exposure to neomycin may result in conformational changes in cell membrane structure leading to disruptions in cell organization and function. Such disruption might render a sensory cell more susceptible to mechanical damage by acoustic exposure.

Alternatively, noise exposure may lead to alterations in the pharmacokinetics of the drug. For example, noise induced changes in general physiological state may affect the distribution, metabolism, or excretion of the drug in animals exposed to high levels of noise. In this case it should be possible to demonstrate higher drug levels in the plasma as well as in perilymph of the noise exposed animals. Another possibility is that noise exposure exerts an effect on the pharmacokinetics of the drug that is localized to the ear (Marques, Clark & Hawkins, 1975). In this case the noise exposed animals might show elevated drug levels in perilymph, but not in the plasma. Further investigation is needed to determine whether any of the above mechanisms participates in the interaction between noise and neomycin reported here.

ACKNOWLEDGEMENTS

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ZUSAMMENFASSUNG

Schädigung der Cochlea durch Neomycin kombiniert mit akustischer Überstimulierung wurde an Meer-schweinchen untersucht. Vier Tiergruppen erhielten 7 Tage lang tägliche Injektionen und wurden Breitbandlärm ausgesetzt wie folgt: I Neomycin (200 mg/kg) mit 10 Stunden Lärm von 115 dB Lautstärke II physiologische Kochsalzlösung mit 115 dB Lärm III Neomycin mit schwachem Lärm (45 dB als akustische Kontrolle) oder IV physiologische Kochsalzlösung mit 45 dB Lärm. Nach einer 30-tägigen Stabilisierungsperiode wurde jedes Ohr elektrophysiologisch durch Messung der Cochlea Wechselstrompotentiale zwischen 100 Hz und 20 kHz und histologisch durch Zählen der äußeren Haarzellen untersucht. Es wurde eine deutliche Wechselwirkung zwischen Neomycin und 115 dB Lärm (Gruppe I) gefunden, die zu einer verstärkten Schädigung führte. Der Empfindlichkeitsverlust der Cochlea betrug im Durchschnitt von allen Frequenzen 62 dB. Der durchschnittliche Verlust in den Gruppen II und III war nur 18 dB und 17 dB. Der Verlust an äußeren Haarzellen betrug fast 100% in Gruppe I, während nur 17% in Gruppe II und 26% in Gruppe III verloren gingen.

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PATULOUS EUSTACHIAN TUBE

Diagnostic Evaluation by Sonotubometry

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Abstract Twenty five patients (31 ears) with pathologically patent Eustachian tubes are presented and the symptoms are analysed. All the ears were examined by sonotubometry and two different opening patterns could be separated for the patulous tube. The variations in impedance of the ear during respiration were recorded from nearly all ears and three ears with eardrum perforation were also investigated by means of the airpressure equalization method. Only sonotubometry brought out clearly the patulous Eustachian tube in all the ears examined. It is thus a useful addition for diagnosing the abnormal patency of the Eustachian tube and for following up the results of its treatment. Then the explanation of the cure does not remain open to conjecture.

The middle ear is normally aerated when the Eustachian tube is opened by the combined action of levator and tensor veli palatini muscles during swallowing. The most common malfunction of the auditory tube is the inability of the tube to open and less commonly, the failure of the tube to close. However the latter, a patulous Eustachian tube is probably not always recognized and may be easily underdiagnosed and mismanaged.

Several authors (Perlman 1939, Moore & Miller, 1951, Suehs, 1960, Miller, 1961, Flisberg & Ingelstedt, 1970, Munker, 1977) have reviewed in detail the symptoms and etiology of the patulous tube. If the physician is familiar with this disorder and the signs and symptoms present the diagnosis will be easily established. The diagnosis can even be discovered by the characteristic history of the patient alone. The feeling of the occlusion or pressure, the hearing of one's own voice and respiration sounding louder than usual and the

sensation of the head as in an empty barrel or in a tunnel (echoing) are the usual complaints about the ear concerned. The symptoms may be relieved during an upper respiratory tract infection, by lying down, by bending the head forward between the knees, or by a sharp inspiratory effort through the nose with nose and mouth held closed (sniffing) or performing the reverse Valsalva manoeuvre. Moreover, on examination the tympanic membrane can be observed to move in and out synchronously with respiration.

The symptoms may also be intermittent or recurrent and findings may be normal on examination and the diagnosis may remain in doubt. On the other hand, the tube may be patulous without giving rise to any complaints. However, as a rule an abnormally patent Eustachian tube causes the patient annoyance and anxiety, sometimes even more than the periodically obstructed tube.

In the present paper the results of a new method of examination—sonotubometry (Virtanen, 1977 1978)—are reported as applied for the diagnosis of the patulous Eustachian tube.

MATERIAL AND METHOD

The material is composed of 25 patients (31 ears) with some of the following present or anamnestic symptoms and signs of the patulous Eustachian tube: feeling of occlusion or fullness in the ear, hearing of one's own voice (autophony) and/or breathing directly in affected ear, relief by lying or sitting.

down and/or the moving of the eardrum synchronously with respiration (examined by Siegle's otoscope). The ages ranged from 27 to 83 years with a mean age of 40, in 15 women and 10 men. 21 patients (27 ears) had intact eardrums and in 13 cases (16 ears) out of these there was a negative history of ear disease. Three patients (3/25) had a dry tympanic membrane perforation, and no signs of infection were evident in the ears and one (1/25) subject had an atrophic eardrum. All subjects were normal on examination of the nasal cavity, pharynx and rhinopharynx showing no definite abnormality and the orifice of the Eustachian tube appeared normal on both sides.

The Eustachian tube function was determined by the sonotubometry previously described in detail (Virtanen, 1977, 1978). The main principle of this method is to introduce the pure tone of 6, 7 or 8 kHz through the nasal olive tip into the nostril. The test sound passing through the Eustachian tube during tubal opening is picked up by the microphone embedded in the circumaural ear defender and fitted with a probe of suitable size in the external ear canal. The amplified output of the microphone is fed through the narrow band-pass filter in order to suppress background noise and is recorded by a level recorder in dB SPL re 20 μ Pa.

For the sake of comparison the three ears with tympanic membrane perforation were also investigated by means of the air pressure equalization procedure according to Flisberg et al. (1963) and Holmquist (1969) with some modifications. This ventilatory function of the Eustachian tube was studied by means of an electroacoustic impedance bridge (Teledyne Model TA 2C) the manometer section of which was used. A negative pressure level of -200 mmH₂O was produced by means of the air pressure pump of the impedance meter into the external auditory canal and the middle ear. The patient was then asked to swallow a sip of water repeatedly as a rule ten times. The equalization of the negative pressure induced into the middle ear occurred in stages either

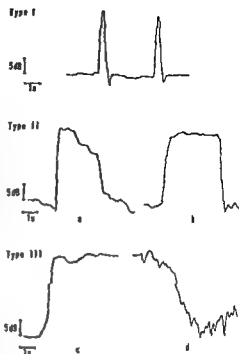


Fig. 1 Different sonotubometric wave patterns. For further details see text.

fully or partially to a certain level and this residual negative pressure was recorded.

A tympanogram was made and the impedance changes caused by forced respiration through the nostril at the examined side were also recorded by the above mentioned impedance measurement equipment in most cases. The hearing was estimated with the pure tone audiogram.

RESULTS

Nearly all the patients (24) had abnormal functioning of the Eustachian tube as examined by sonotubometry. The tubal opening recordings measured in the frequency of 7 kHz were divided into three groups according to the nature of opening. Fig. 1 shows samples of the different sonotubometric wave patterns.

Type I function is characterized by a relatively sharp peak of increase in the sound pressure level during swallowing. It describes the normal finding of the Eustachian tube function (Virtanen 1977, 1978).

Table 1 Twenty five cases of patulous Eustachian tube

Age	Sex	Symptoms in ear				Results of examination			
		Sensation of fullness	Autophony	Respiratory sound	Relief by lying down	Drum moves with respiration	Sonotubometric curve type	Variations in impedance with respiration	Residual negative middle ear pressure (mmH ₂ O)
48	♀	+	+	+	-	-	I		
70	♀	+	+	+	+	-	II		
73	♂	+	+	+	+	+	II		
57	♀	+	+	+	+	-	II	-	
36	♀	+/-	-	-	-	+	II	+	
46	♀	-	+	-	-	-	II	+	
38	♂	+	-	-	-	+	II	+	
52	♀	+	+	+	+	+	II	-	
77	♂	+	-	-	-	+	II	+	
35	♂	+	+	+	+	+	II	+	
40	♂	+	+	+	-	+	II	+	
29	♀	+	+	+	+	-	II	+	
36	♀	+	+	-	+	-	II	-	
30	♀	+	+	+	+	+	II	+	
31	♀	+	+	+	+	-	II		
46	♂	-	-	-		perfor	II		-100
27	♀	-	-	-		perfor	II		II
38	♀	+	+	-	+	+	III		
51	♀	+	+	+	+	+	III	+	
53	♀	+	+	+	+	+	III	+	
78	♂	-	+	+	+	-	III	+	
38	♂	+	+	+	+	+	III	+	
27	♂	+	+	+	+	+	III	+	
78	♀	+	+	+	+	+	III	+	
30	♂		-			perfor	III		-200

the second type (II) the tube opens very little on swallowing and for some time afterwards and closes little by little (Fig. 1a) or abnormally with or without further swallowing (Fig.

the third type (III) the Eustachian tube opens well on swallowing and remains open for a long time or is continuously open (Fig. 1b) and it may be closed by bending the head backward (Fig. 1d), by lying down and/or by blowing.

Table 1 shows the analysis of symptoms and findings related to the tubal function for all cases. In 6 patients both ears were involved. In one person's (patient 5) symptoms varied from ear to ear. The results of examination were the same on both ears of all these 6 patients. Of the first 17 patients are those who showed type II of sonotubometric curve except one (patient 1) who had the normal sono-

tubometric curve. The fact that she had had a common cold a week ago may be taken into account and probably the swollen mucous membrane was still resulting in a normal tubal opening. Two patients (16 to 17) had a dry, central tympanic membrane perforation.

None of the 15 patients with intact eardrums had the symptoms of the patulous tube continuously but at most for a few hours a day and not always every day. Three patients did not feel the sensation of occlusion or fullness of the ears, 3 out of these (15) were not annoyed by autophony and 6 patients did not hear their own respiration in their ears. Only 9 patients obtained relief by lying down and placing the head down between the knees. In 8 patients the drums were seen to move (examined by Siegle's otoscope) with respiration at the commencement of the examination. According to the sonotubometric curve the

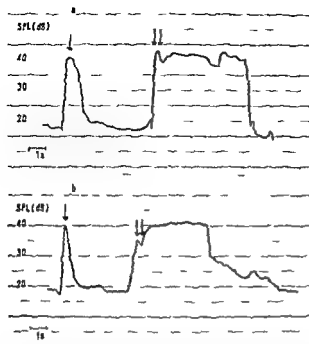


Fig 2 Two individual sonotubometric examples of tubal opening during swallowing (I) and by means of yawning (II). Upper curve (a) the patent tube is closing voluntarily and abruptly after yawning, and lower curve (b) II is closing little by little by bending the head forward

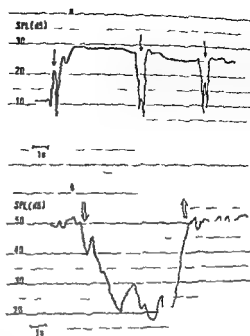


Fig 3 The individual sonotubometric curves of 2 patients. The first arrow (I) is the start of the test and the second arrow (II) is the end of the test. The thick arrow mark the direction of head moving

tachian tubes in these 17 ears opened after swallowing for the time of 1-13 sec closing little by little or abruptly (Fig 1, type II). Ten of the first 17 patients were able to open their tubes easily by means of yawning and to close them voluntarily (Fig 2a) or by bending their heads forward (Fig 2b).

Nine patients (8 to 16 Table I) had had otitis media previously, and even tympanostomy tubes were used in one patient. One patient (17) had had a fracture of the base of the skull as the result of an accident.

In the remaining 8 patients (18 to 25) the sonotubometric curves were of the type III (Fig 1), i.e. the Eustachian tube was open continuously or for a long period of time and did not close during swallowing (Fig 3a). Of these patients with intact eardrums only one stated that the ear was not occluded. Each one of these patients heard his own voice (autophony) and all of these patients were able to

obtain relief by lying down or bending the forward (Fig 3b). One patient did not hear his own respiration in the ear and in one patient drum movements with respiration were not seen. Of the 8 patients with type III sonotubometric curve, 4 had learnt to get rid of symptoms by means of a quick inspiration through the nose (sniffing) intermittently (Fig 4a), whereas some did not succeed in the attempt to close the tubes by sniffing (Fig 4b). Three (18 to 20) out of the remaining 8 patients had a history free of ear disease and had had otitis media several times in the past or more recently, tympanostomy tubes were even needed for one patient. One patient (21) had had a fracture of the base of the skull but no ear trouble prior to that.

None of the 3 patients (16, 17 and 25) with an eardrum perforation had any symptoms of a patent tube. The pressure equalization was also made in these three ears. One

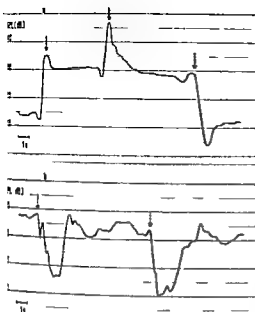


Fig 4 Two visual sonotubometric curves of the ear

using (a) Lower curve (b) the continuously patent tube

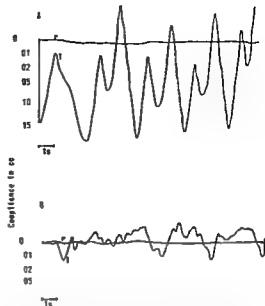


Fig 5 The variations in impedance of the ear with respiration on two different patients (a=right ear normal tubal function b=left ear patulous tube) For further details see text

able to equalize the negative middle ear pressure partially to levels of $-100 \text{ mmH}_2\text{O}$ only. The other was not able to equalize it at all. However both these ears had an abnormally patent Eustachian tube on examination by sonotubometry the types of curves were II and III. The third ear with tympanic membrane perforation was able to normalize the negative pressure introduced into the external ear canal and it had sonotubometric curve II. Four patients (18 to 20 and 24) reported too that they often suffered from a sensation of occlusion in the nose and/or a nasal discharge. In one patient even allergy alone was suspected as being the main cause. The otologic examination was essentially negative and it revealed free nasal passages in all these patients. Two patients' voices were heard to be decidedly peronasal but the phoniatrician did not recognize any disturbance of the velar function or articulation.

The hearing level of all these ears with intact eardrums was within 20 dB of the ISO standard reference threshold at the frequency range of 500–2000 Hz. They had as a rule the normal shape of tympanogram too. With the aid of an impedance measurement the variations in impedance could be observed synchronously with respiration in nearly all patients. The amplitude varied greatly not depending in any way on the kind of tuba aperta that existed as shown in Fig 5 where the patient A (10) had the sonotubometric curve type II and the patient B (19) had the curve type III denoting the continuously open tube.

COMMENT

There are many people who have patulous tubes and who suffer from the symptoms either continuously or periodically (Munker 1977). The symptoms are variable because the patency is not always constant and it may vary from a mild unilateral form to a constantly open bilateral form.

The symptoms in this material also varied a great deal, and thus the diagnosis of tuba aperta could not be reliably founded on them alone. The patients with a tympanic membrane perforation did not suffer from the symptoms of the patulous tube at all. This is in accordance with the suggestion of making a myringotomy with tube insertion into the middle ear in order to offer improvement or complete relief (Succes, 1960, Thaler & Yanagisawa, 1966).

The analysis of the sonotubometric findings of this work showed that the patients with a patulous Eustachian tube had some typical characteristics in their wave patterns (Fig. 1). Thus, apart from one patient, the diagnosis could be confirmed by means of the sonotubometry. This patient did not have a patulous pattern of tubal opening during swallowing which evidently was due to a nasopharyngitis that she had had just a week before.

By measuring the variations in impedance of the ear during forceful respiration the diagnosis might be established, as described earlier by Metz (1953) too. However, the movements of the tympanic membrane are not always present at the moment of the examination and thus a false negative result can be obtained, as on 4 patients in this material. Measurements of impedance variations did not correlate well with the results of sonotubometry concerning the type of the patulous tube.

In two ears out of these three with an eardrum perforation the pressure equalization test was inapplicable to the evaluation of tubal function. Only one patient could equalize the negative intratympanic pressure fully, even though all the tubes were abnormally open. It is evident that the pressure equalization test does not give correct information about the patency of the tube, because a negative middle ear pressure can cause the locking phenomenon of the tube and produce an obstruction that the muscle activity of the tube is not able to overcome (Virtanen 1977, Palva et al., 1978). Thus on the ears with a drum perforation the sonotubometry alone gives a quite

distinct objective impression of the permeability of the tubes. It may be worth noting because after the healing of the eardrum symptoms of the patulous tube are more likely to affect these persons, who have a deficiency in the closing of their Eustachian tubes. According to Ekvall (1977) these patients often try to close their tubes by sniffing and thus cause a medial displacement of the eardrum.

Two patients (17 and 21) were injured as a result of an accident, with a fracture of the base of the skull passing through the ear. They had had no ear trouble before; thus the fracture was the apparent etiological factor and the pathological basis for their condition.

In this material there were 9 (36%) patients who had suffered many otitis attacks and concurrent middle ear effusions or discharges in their previous history. On the basis of this etiology of the recurrent otitis media could some of the cases be attributable to the failure of the Eustachian tube to close for considerable periods of time. Later, by following the infection of the middle ear, structural changes may develop on the mucosa of the Eustachian tube and the tube can then be partially or totally obstructed and the need for prolonged use of tympanostomy tubes may arise (cases 11 and 22). Aschan et al. (1964) assumed that the sniffing repeated continually several times a day, may be the cause of atrophic tympanic cavities in these patients but this will require further investigation.

Three patients had feelings of discharge and obstruction in their nasal cavity similar to the symptoms of a common cold. One patient of these 3 had been suspected of allergic rhinitis and had been examined thoroughly but with negative results. None of these had the nasal obstruction and distinct discharge from the nose at the examination. All their tubes were open continuously or for long periods of time (sonotubometric type III) they were perpetually trying to sniff. Two patients had inadvertently learnt to avoid nasal respiration preventing by velar fu-

nasal resonance from extending through tube into the middle ear. Similar hypotality of speech caused by the patulous tube also been described before by Landes (67), and Batze & Parker (1971).

ZUSAMMENFASSUNG

werden 23 Patienten (31 Ohren) mit pathologisch klapf des Ohrtrompeten vorgestellt und die Symptome analysiert. Alle Ohren wurden mit Hilfe der Sonotubometrie untersucht und es konnten zwei verschiedene Öffnungsweisen bei der offenstehenden Tube unterschieden werden: Schwankungen der Impedanz des Ohres bei der Resonanz wurden in fast allen Ohren aufgezeichnet, und 10 Ohren mit perforiertem Trommelfell wurden auch mit Hilfe der Luftdruckausgleichsmethode untersucht. Nur Sonotubometrie zeigte deutlich die offenstehende Tube in allen untersuchten Ohren. Sie ist somit eine brauchbare zusätzliche Methode zur Diagnose des abnormen Öffnens der Ohrtrompete und zum Verfolgen der Behandlungsergebnisse, wobei dann die Beurteilung der Handlung keinen Raum für Vermutungen läßt.

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TUBAL CLOSING FAILURE IN RETRACTION TYPE CHOLESTEATOMA AND ADHESIVE MIDDLE EAR LESIONS

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Abstract In cases of retraction type cholesteatoma and related conditions it has been observed that the Eustachian tube may show a reduced ability to withstand respiratory pressure variations. The reduced resistance of the tube may play an important role in the development and course of middle ear disease. In the present study 42 subjects who had middle ear disease of this type and confirmed habitual sniffing behaviour were examined for symptoms and signs that would indicate a patulous tube. Different degrees of malfunction were recognized in the presence of total closing failure with an intermittently free transfer of respiratory pressures to the middle ear, the sniffing procedure maintains a negative intratympanic pressure. The condition is identical to the patulous tube state in the classical sense. In *relative* closing failure there is no free pressure transfer and no patulous tube symptoms. Sniffing may however cause a repeated evacuation of the middle ear space. The results of this study cast doubt upon the conventional views on tubal malfunction in middle ear disease and indicate a need for reconsidering the etiology of these lesions.

The physiological role of the Eustachian tube is usually thought of in terms of different components:

(1) Ventilation of the middle ear space and of the air cell system is regarded to be the most important function. The tube is opened briefly by muscular contraction during deglutition, permitting an intermittent passage of air. Any volume deficit caused by the gas exchange in the middle ear, and any change of atmospheric pressure, are thus compensated for. A pressure difference between the lateral and medial sides of the tympanic membrane is thereby eliminated.

(2) In the passive, closed position the tube protects the middle ear against sudden pressure changes in the nasopharynx caused by

respiratory activity and phonation. If closed, the tube also protects the middle ear against infection which might ascend from the nasopharynx.

(3) The mucociliary function takes care of clearance of mucus and foreign material including bacteria.

That a close interrelation exists between different components of the Eustachian tube function is obvious but investigations of this function have dealt almost exclusively with ventilation with respect to anatomical and functional patency. An impaired ventilator ability is believed to be a contributing factor in the development of different diseases of the middle ear. It is also believed to be causative in failure of healing after reconstructive ear surgery, and in recurrent cholesteatoma. Clinical tests used at present are based on the assumption that the capacity to equalize artificial pressures is crucial. For example the aspiration-deflation method described by Fritsberg (1966) has been used to a large extent with individual variations in the evaluation of "tubal function". Current methods have recently been reviewed by Cohn (1977) who questions the relevance of the aspiration test as far as the physiologic function of the tube is concerned and concludes that the test is not suitable for predicting the outcome of tympanoplasty. This is in agreement with the findings of Ekvall (1970) and Palva & Kari (1970).

When a suitable surgical technique is used in myringoplasty, there is no correlation

ween the aspiration test result and postoperative healing. There is thus an obvious need for examining the tubal function test. To test the normal function of the tube under realistic circumstances may actually call for a rather different approach, an integral part of which would be to remove much of the present emphasis on the ventilatory capacity.

Closing Action of the Tube

According to Elner (1977) the function of the tube is not ventilatory in a strict sense. The extremely small quantities of air which normally pass through the tube do not change the gas composition of the middle ear space significantly. A better definition of the function of the tube would be pressure regulation, or pressure control. Considering the rather abrupt respiratory pressure changes which take place in the nasopharynx, it seems more apt to focus on the closing ability of the tube.

The patulous tube condition has been referred to in a number of papers. Since the condition involves an apparent "hyperfunction", it has been set clearly apart from the tubal problem in middle ear disease. A good review of the literature and of the symptoms of this condition was published by Miller in 1961. In his material and in others, are included several cases of atrophic tympanic membranes. Miller concludes that the state is much more common than is appreciated, depending to a great extent on the difficulties which the patients have in describing their symptoms.

Moreover, the patulous tube state is easily confused with its apparent opposite, the stuffed tube with serous otitis media. It should be noted here that the two clinical entities are perhaps not mutually exclusive. Munker (1977) has noted a patulous tube in many cases of cleft palate, and Zollner (1942) has often observed a patulous tube in cases of both serous otitis and chronic otitis media.

In persons with a patulous tube, negative middle ear pressures are created by repeated, forceful nasal inhalations, forcing the tube to

close. It is well known that such persons very often use a sniffing procedure to suppress their symptoms. Possibly, the self induced underpressures might cause structural lesions in the middle ear.

The following study was initiated as a result of a clinical observation. A young woman of 28 had bilateral attic cholesteatomas, collapsed tympanic membranes and effusions in the middle ears. In the preoperative period the patient was instructed to ventilate her ears actively by Valsalva inflations. She refused firmly. Instead, it was discovered that she performed reverse Valsalva manoeuvres continuously in order to avoid autophonia, respiratory drumhead flutter and hyperacusis. In spite of great efforts at persuading her, the patient still refused to try Valsalva inflation. It was eventually possible to convince her to abandon the reverse Valsalva manoeuvres, however. From that day, both middle ears became spontaneously air filled without effusion, and her hearing has remained good for the 5 subsequent years.

In this case there was evidently a defective closure of the tubes, which had caused the patulous tube symptoms and the reactive behaviour. The opening action of the tubes proved to be sufficient when the self induced evacuation of the middle ear space was omitted. The same impairment was soon found in several other cases having middle ear disease of similar type. Ultimately, it was suspected that a direct, causal relationship might exist between the failure of the tube to close and the development of chronic middle ear disease, and that the relationship might constitute a generalized etiological principle. In the present study the attention is focused on the reduced ability of the tube to remain closed in the resting position. These aspects have been presented preliminarily by Ekvall & Magnuson (1976, 1977), Magnuson & Ekvall (1976), Aschan, Ekvall & Magnuson (1977). This study is a systematic analysis of anamnestic data and otomicroscopic findings in a selected material, easy to adapt in clinical practice.

Table I

Healthy ear	4
Healthy ear post comb appr tympanoplasty	3
Recurrent serous otitis media only	2
Atrophy of pars tensa only	10
Deep retraction of pars flaccida only	8
Deep retraction of pars tensa and pars flaccida	17
Retraction type cholesteatoma not operated	21
Advanced adhesive otitis	2
Perforated retraction	1
Radically operated ear	9
Complication post comb appr tympanoplasty	7

MATERIAL AND METHOD

The present material consists of 42 subjects who were either treated conservatively for middle ear disease, were awaiting surgery, or had previously undergone middle ear surgery on one or both ears. The subjects were selected because they had a pronounced habitual sniffing behaviour. The age range was 14-65 years, mean age 39, median 41.5 years. No young children were included, since they cannot describe their symptoms accurately. 14 subjects were female and 29 were male. In most cases the middle ear disease was bilateral, only 4 had unilateral disease. The diagnoses in all 84 ears at the time of the investigation are given in Table I. It is seen that there were no cases of central perforation in the material.

The lesions range from subtle atrophic changes of the tympanic membrane, to deep retractions, cholesteatoma in retraction pockets, 'spontaneously operated' cases with a wide destruction of the medial canal wall and labyrinthine fistula, and finally to advanced adhesive otitis with a totally obliterated middle ear space, save for a tiny air bubble in the tympanic orifice of the Eustachian tube.

A long history of purulent drainage was present in 26 of the subjects. The severity of diseases in the group may be indicated by the fact that 19 of the subjects had earlier undergone middle ear surgery, a total of 32 surgical procedures and revisions. The material includes 7 cases of recurrent retractions post CAT procedures. One of these had recurrent

cholesteatoma, 2 had a late penetration of the columella through the healed fascia graft and one had a late spontaneous hemotympanum. At the time of the investigation 9 of the subjects were being treated for middle ear effusions by ventilating tubes.

All ears were examined by otomicroscopy, and by simple functional tests. The subjects were questioned systematically about symptoms, provocative factors, and sniffing.

RESULTS OF INTERVIEW

A Autophonia

A blocked sensation in the ear is described by most patients and might easily be mistaken for serous otitis media. These patients frequently have effusions, which might add further confusion. When the patients were questioned in detail, a common picture of a specific type of autophonia arose. One's own voice seems hollow, as if one were speaking into an empty barrel, it seems too loud, roaring and distorted. The nasal speech sounds break through as do the palatal click sounds, and breathing sounds. The symptoms are of very short duration, only a second or two, and are instantly switched off by sniffing. Twenty nine of the subjects had autophonia and 90% of these patients had it in the form of short recurring episodes.

	n	%
autophonia	29	69
no autophonia	13	31
total	42	100

Course of autophonia

	n	%
short recurring episodes	26	90
single episodes	1	3
lasting autophonia	2	7
total	29	100

The autophonia could be evoked by certain factors in 19 of the subjects. The remaining 10

subjects could not indicate any provocative
ors

Provocative factors

Physical strain, like lifting heavy objects,
running playing tennis and soccer 14 sub-
jects mental strain and stress 7 subjects,
environmental temperature change, like going
outside in cold weather 6 subjects

B Drumhead Flutter

Movement of the tympanic membrane syn-
chronously with respiration was a common
symptom in this group of patients. It is de-
scribed as a feeling that the drum is loose and
will not come to a resting position. Clicking
or pumping sounds arise in the ear upon
breathing. The symptoms are both tactile and
acoustic. The flutter is instantly switched off
by sniffing. Twenty seven of the subjects had
drumhead flutter, and in 82% of them it ap-
peared as short recurring episodes.

	n	%
Drumhead flutter	27	64
No drumhead flutter	15	36
Total	42	100

Course of drumhead flutter

	n	%
Short recurring episodes	22	82
Isolated episodes	3	11
Increasing drumhead flutter	2	7
Total	27	100

The flutter could be evoked by certain factors
in 21 of the subjects. The remaining 6 sub-
jects could not associate the symptoms with
any specific events.

Provocative factors

Physical strain 16 subjects
Mental strain 6 subjects
Temperature change 5 subjects

C Hyperacusis

Hyperacusis was described by 17 subjects.
This disturbance is apparently paradoxical in
view of the fact that most subjects had im-
paired hearing with different degrees of air-
bone gap. In many cases it was found that not
only the patient's own voice but also environ-
mental sound was heard too loudly. With
"drums out" after spontaneous equalization
caused by swallowing, the sound appears too
loud, echoing, sharp and distorted. With
"drums in" the hearing is more comfortable,
natural and distinct. Hyperacusis is also of
short duration, and is suppressed by sniffing in
these patients.

D Common Colds

Patients with a patulous tube can generally re-
lieve themselves of their symptoms by lying
down, which was confirmed in all but two. In
the majority of the present cases, the outbreak
of a common cold was not followed by relief
but was often associated with an enhancement
of symptoms, including an increased need for
sniffing. In the late stages of the infection the
autophonia, drumhead flutter and hyperacusis
became less annoying, but at the same time the
hearing was often more impaired than usual.

Symptoms at the outbreak of a common cold

	n	%
autophonia worse	15	52
no change	13	45
improvement	1	3
total	29	100
	n	%
drumhead flutter worse	14	52
no change	12	44
improvement	1	4
total	27	100

E Voluntary Suppression of Symptoms

Voluntary suppression of symptoms by means
of sniffing was claimed to be habitual and to
date back as long as the subject could re-

member in 25 of the 42 subjects in this material, or 60%. Occasional sniffing was claimed by 13 subjects, 30%. Four persons said they were not conscious of any sniffing, though an obvious sniffing behaviour could be observed.

In 29 cases sniffing only was used, and in 7 cases it was used in alternation with reverse Valsalva manoeuvres. In 6 cases, only reverse Valsalva was used to suppress the symptoms.

F Reasons for Sniffing Procedure

Several subjects gave more than one reason for their sniffing habit.

	n	%
no reason	5	12
autophonia	25	60
drumhead flutter	20	48
hyperacusis	17	40
noise protection	12	29
to hear better	16	38
other reasons	8	19

Among 'other reasons' was the need to relieve high frequency tinnitus, which was mentioned by 2 subjects. As possibly contributing factors were allergic rhinitis or other nasal problems in 11 cases, and significant occupational noise exposure in 21.

G Active Ventilation for Hearing Improvement

The majority of the patients studied were able to increase their hearing acuity by Valsalva inflation when listening to weak sounds. Not all utilized this possibility, however. Thirty-three stated that they could improve their hearing by inflation, but only 17 were inclined to do so. When evaluating this finding it should be noted that all subjects had been instructed earlier and motivated repeatedly to carry out active ventilation in connection with the treatment of hearing loss and middle ear effusions. When interviewed in the present study, 50% claimed to be unwilling to perform ventilation.

Several subjects stated more than one reason for their unwillingness.

	n	%
no statement	18	43
ventilation would bring on		
autophonia	14	33
drumhead flutter	1	2
hyperacusis	12	29
pain	3	7
other reasons	7	17

Other adverse effects experienced by subject to such an extent as to render them unwilling to carry out ventilation were sudden vertigo in 2 cases and painful pressure in the eyes in 1 case.

RESULTS OF CLINICAL EXAMINATION

A Valsalva Inflation

Patients were instructed to perform Valsalva inflation under visual control via the otomicroscope. As mentioned, some subjects were unwilling to perform inflation. This was especially true of the younger individuals. The patient's attitude toward performing Valsalva inflation and, once willing, his way of doing so provided fairly good indicators of the presence of closing failure. Some persons merely mumbled the inflation by keeping the glottis closed during the procedure. It was usually very easy to obtain passage when the right technique was used. Even when assuming a supine position on the examination table, the patient could generally perform the inflation without ease. In this position many normal subjects have difficulty in inflating their ears. Characteristic for the present patients was that the tympanic membrane first bulged due to the increased pressure, and then collapsed again. It seems that the tube cannot maintain any positive pressure within the middle ear in these cases. In 10 ears the presence of a ventilation tube or a perforation made the test irrelevant.

Passage in Valsalva inflation

	n	%
negative	6	8
positive great resistance	7	10
positive easy passage	6	8
positive easy passage in supine position	55	74
total	74	100

II Sniff Test

The sniff test was carried out during otomicroscopic examination. After Valsalva inflation the subject is asked to occlude one nostril with the finger and to perform sniffs with increasing force. If the tympanic membrane is seen to retract instantly, the test is considered positive. Sometimes the drumhead flutter caused by slow or forced respiration may be observed. Often the subject has to sit up in order to perform the Valsalva inflation. If he still does not succeed a Politzer inflation is performed. The subject is then asked to lie down on the examination table again, and told to make sure that he does not sniff until the microscope has been focused. Sometime the test is positive in the sitting position but not in the lying position. It is important that the subject does not spend unnecessary time in the supine position. The increased hydrostatic pressure makes the tube close very rapidly. In the presence of a perforation, a ventilating tube or a stiff and tympanosclerotic tympanic membrane the test is not relevant. In the present material the test was irrelevant in 17 ears.

Result of sniff test

	n	%
positive test	59	82
negative test	13	18
total	72	100

CLINICAL PRESENTATION OF CLOSING FAILURE

A typical case in this material is a 40 year old male subject with bilateral attic retractions

or cholesteatoma, a patulous tube condition and a confirmed habitual sniffing habit for many years. Most of the subjects stated that they had had the symptoms, as well as the sniffing habit, for as long as they could remember. Some of the subjects could give lucid accounts of childhood experiences regarding these matters. The patulous tube condition thus does not seem to be limited to advanced age, weight loss, hormone treatment or other general conditions. Erect posture and stress situations of various kinds seem to provoke the symptoms in the present cases.

A pronounced variability between different patients is indicated by the present findings. Furthermore, when subjected to repeated examinations, one and the same individual will display different impairment pictures. On one occasion, a total closing failure with a free respiratory drum movement could be found. A common cold may cause the tube to be totally blocked, and a few weeks later the closing failure may turn up again. There is thus a very pronounced intra individual variation. The malfunction is not a static condition, but rather, rapid change seems to be inherent. The tubal malfunction may be described by its degree and duration, and all combinations seem to be possible. The term 'tubal closing failure' may then serve to cover various conditions involving an impaired resistance of the tube. Some systematization may be supplied by dividing the conditions into the following clinical groups.

A Total Closing Failure
Constant Type

Such cases are detected accidentally, since there are no symptoms. A person can obviously get used to this type of closing failure. At repeated examinations the drum is seen to move synchronously with respiration, a manifest condition of a wide open tube which may be detected easily by otoscopy. If no progressive middle ear disease is present, there is no need for treatment. In order not to evoke

his anxiety, the patient should be informed that the findings do not indicate disease

B Total Closing Failure, Intermittent Type, Non suppressed

This condition is met with more commonly, since the patient is alarmed by the intermittent onset of symptoms. Because positive findings may not be present at the time of examination, the nature of the disorder is easily misinterpreted, and the patient may seek medical advice repeatedly. The condition seems to produce very pronounced symptoms, by virtue of its being intermittent. Habituation is therefore not possible.

There are difficulties in tuning the voice to a suitable loudness level. When the tube is open, the autophonia causes a distressing and constantly varying error in the natural feedback of speech and hearing, which may lead to a severe neurosis. Drumhead flutter, the feeling that the tympanic membrane moves and flutters as one breathes is also trying. The membrane may move in a distressingly irregular manner, depending on changes in depth and velocity of respiration. Most of these cases can be diagnosed easily if attention is paid to the case history.

Information to the patient on anatomy and function in common terms is the primary treatment. This provides a rational explanation for the symptoms, and will often relieve the patient's anxiety. The symptoms are evoked by stress situations and are usually of only some minutes' duration. When told that such symptoms are quite common and that they do not indicate disease, the individual will tolerate them more readily. For the most part no other treatment is necessary. In the case of more protracted symptoms, the insertion of ventilating tubes may eliminate the symptoms or relieve them significantly.

C Total Closing Failure Intermittent Type, Suppressed

This condition constitutes the main issue of the present paper. Sniffing is used habitually

to suppress the symptoms. This procedure is well known in patulous tube cases but the relation to retraction type cholesteatoma does not seem to have been recognized. By a forceful nasal inhalation or a reverse Valsalva manoeuvre a low middle ear pressure is induced and is maintained for some time because of the valve action of the tube. The subject is thus briefly relieved from his symptoms when the tube is locked. Upon swallowing the tube may easily open again causing the autophonia to reappear. The good equalizing ability of the tube is thus maintaining the state and the events are repeated in a cyclic fashion over and over again.

Presentation

In the present cases the sniffing habit seems to have been acquired early in life. The subject has found a way to cope with his symptoms and he usually seeks medical care not because of autophonia, but because of middle ear disease. However, this is often not discovered until specific symptoms of chronic otitis media arise. Hearing frequently remains good and discharge, pain and other complications are late symptoms. At the time of admittance the impressive otoscopic findings attract attention. The specific tubal malfunction is not expected and is therefore easily overlooked. Furthermore, the symptoms may have been present for many years, but habitually suppressed by sniffing on an everyday basis. Hence the patient may not even be aware of any symptoms himself.

Symptoms

Many patulous tube cases have normal hearing according to routine audiometry. Even so the subject experiences a severely disturbed distorted hearing. When questioned some can give a full account of their symptoms while others merely describe a vague feeling of uneasiness. Upon swallowing or yawning a popping sound may be experienced and the ear is said to feel 'blocked'. The automatic reaction of the individual is to sniff. The patient

is a very strong desire to make the drums come to a stand still in the retracted position and the hearing is said to be better after sniffing

Objectively an impaired hearing exists in the majority of the present cases but hearing is not a primary complaint. The patient may find hyperacusis to be the most disturbing symptom. Sometimes it is the only one. A probable explanation for this paradoxical symptom is habituation. These subjects are constantly sniffing in order to suppress the autophonia and drumhead flutter and seem to accept a certain impairment of the hearing caused by the low intratympanic pressure. As they become more and more used to it they come to consider it as their natural hearing. By adaptation to the experience of loudness may transform itself into a symptom of its own. With drums out the subject experiences environmental sounds as strange, unpleasant, and sharp and non clear. When the sound source is changed suddenly by spontaneous equalization a compulsory stimulus for a sniffing reaction is provided even though the subject may be free from autophonia at the time.

The condition is especially troublesome for the subject who has a noisy occupation. If he has to speak loudly or to shout in order to make himself heard the autophonia and hyperacusis become intolerable. A certain constructive impairment is advantageous in a noisy environment and in the case of closing failure the subject makes use of his unique ability to protect himself. Sniffing is the only way to master the situation.

Obviously a vicious circle develops where secondary conditioning makes the subject sniff increasingly even during periods when there is no total closing failure and no autophonia. These subjects are therefore approaching the next category of relative closing failure.

Diagnosis

The presence of intermittent autophonia, drumhead flutter and hyperacusis as well as

the sniffing habit may be revealed if the patient is questioned in detail. In many cases the diagnosis is apparent at the first visit if attention is paid not only to the middle ear disease but also to a careful penetration of the patient's symptoms and to observing his behaviour during the examination. The sniff test should be carried out routinely and repeated examinations are necessary whereby many of these cases may ultimately be diagnosed objectively.

Treatment

How to treat closing failure as such is not settled although different methods have been tried in the past. Treatment should be aimed at the causal factor which is still unknown. It might be a defect in the tubal secretion which might not be sticky enough to keep the mucous surfaces of the tubal slit firmly glued together in the closed resting position. It seems that viscous fluid is very effective in blocking the tube while serous fluid is not. In the majority of the present cases there is retraction of the tympanic membrane and middle ear effusions have been observed in several. Typically the effusion is thin and watery with some air bubbles anteriorly near the tympanic orifice of the Eustachian tube. It might thus be possible to classify the common serous otitis media together with the present cases.

The appearance of a viscous effusion a perforation of the tympanic membrane or an interruption of the ossicular chain might contribute to extinguishing the sniffing by relieving the patient from patulous tube symptoms. There is some evidence that some patients suffering from recurrent effusions are actually utilizing this effect by sniffing vigorously until the appearance of effusion relieves them from symptoms. The lesion is induced and maintained actively and spontaneous healing is counteracted.

Because of the confirmed reactive behaviour treatment is definitely no easy task. In some cases it is possible to get the patient out of his habitual sniffing. He will find that the

patulous tube symptoms are present for short intervals only, e.g. in the presence of physical or mental strain, temperature change, a common cold or other circumstances of stress. He may find that when the sniffing is avoided the symptoms are not so very disturbing after all. However, in many cases it is seemingly impossible to convince the patient to abstain from his sniffing habit.

The insertion of ventilating tubes is the only simple and instantaneously effective treatment available. The tubes will cause a rapid disappearance of effusion, render underpressures impossible, often relieve the patient from his patulous tube symptoms, and also seem to influence the patient's sniffing behaviour. The hyperacusis may be exaggerated during the first few days and ear protectors may have to be used for some time in order to attenuate environmental sound. Unfortunately, a relapse into the old habitual sniffing behaviour seems to take place very easily when the tubes are extruded.

D Relative Closing Failure

This term might be used to denote a state where the respiratory pressure variations are not freely transferred to the middle ear space. The tube is thus not patulous in the strict sense of the word. Negative nasopharyngeal pressures are given passage, while attempts to ventilate the middle ear by Valsalva inflations may meet with great difficulties. The pronounced one-way valve action of the tube seems to be decisive. In apparent cases the valve action is extreme enough to make Valsalva inflation impossible. A nasal inhalation, without being forced, may induce a retraction of the tympanic membrane. In less obvious cases the tube appears semi-blocked, and only when the tube is subjected to more excessive negative pressures does a pressure break through occur.

Evidently, a habitual sniffing behaviour is not developed in such cases nor is it a necessary precondition for maintaining a nega-

tive middle ear pressure. The pressure may be reduced occasionally, e.g. during snoring in common cold. At present, relative closing failure seems to represent a wide and rather unknown field. It might be a very common state but it is a most difficult condition to diagnose objectively. No acoustical symptoms accompany the relative closing failure and diagnosis cannot be made from the case history. The subject may have a positive snifftest occasionally, but the state seems to vary from time to time just as the two previous ones do.

Relative closing failure has been found in some cases of glue ears and also in most advanced cases of chronic otitis media. Instructing the patient to perform Valsalva inflation intermittently seems to be successful treatment in some cases. However, when the inflation is performed, air may escape spontaneously, because the tube cannot maintain positive pressure within the middle ear. The use of ventilating tubes is frequently necessary, especially in children.

It should be noted that although the middle ear disease is usually bilateral the severity of the disease frequently takes on quite different manifestations. Not infrequently the least affected ear is found to have a closing failure of the suppressed C type, while the most severely diseased ear is found to have a totally blocked tube at the time of examination. The functional defect of the tubes in these cases is thus asymmetrical, seemingly of opposed types. In such cases there is some evidence to indicate that one ear may be the 'leading one' causing intermittent patulous tube symptoms as well as habitual sniffing. The 'second ear' where a closing failure is demonstrated upon examination might be influenced by the sniffing and be blocked because of it. A relative closing failure may thus be present. It has been observed that a ventilating tube inserted in the 'leading ear' may improve the state of the 'second one'. Such information might be of significance in surgery for chronic middle ear disease.

CONCLUSIONS

patulous tube state is not a uniform or static condition. When analysed more closely, it is found to give rise to quite different clinical pictures, some of which will elude diagnosis if they are not searched for actively. It might be found that the tubal malfunction of interest in chronic middle ear disease is a failure not of opening but of closing.

In the present cases the middle ear space is vacuated from the nasopharynx and, in a strict sense, the tympanic membranes are retracted because the subjects retract them. The questions are why and how do they do so, what are the consequences, and what is the significance of this in a non-selected population? In this paper the patients' own accounts of their experiences are used as point of departure. The present material was included in an investigation in which objective measurements were carried out by pressure recording. The results will be dealt with in a paper to follow.

The theory advanced here is that chronic adhesive middle ear lesions and retraction type cholesteatoma may develop as a reaction to certain types of tubal closing failure. The higher pressures which are induced may be responsible for retraction and atrophy of the tympanic membrane, effusion of fluid, and a compressive atelectasis of the middle ear space with involution and sclerosis of the mastoid bone and cell system. By permitting the accumulation of keratin debris in retraction pockets the disease process may be indirectly responsible for the development of cholesteatoma. Since the disease starts early in life, the original tubal malfunction will not necessarily be permanent. If irreversible damage to the tympanic membrane has been induced, the development of cholesteatoma with secondary infection and further damage to the ear may proceed autonomously. In certain cases the tubal malfunction may remain or reappear, leading to a tendency to postoperative effusion, new re-

tractions of the grafted tympanic membrane, and recurrent cholesteatoma.

ZUSAMMENFASSUNG

Bei Erkrankungen des Mittelohrs hat man sich früher ganz auf die Öffnungsfähigkeit der Tube konzentriert. In vorliegender Arbeit wird im Gegensatz die Schließungsfähigkeit der Tube bei Retraktionscholesteatomen und ähnlichen Zuständen als krankheitserregend dargestellt. Vier verschiedene Formen des Funktionsdefekts sind definiert, welche klinische Variationen von klassischer Tube in klassischer Bemerkung darstellen. Über Resultate von Druckmessungen in Retraktionsfällen wird in einem folgenden Artikel berichtet werden.

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SUDDEN DEAFNESS IN RELAPSING POLYCHONDritis

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Abstract The left ear of a 57 year-old female who suffered sudden deafness during the course of relapsing polychondritis was examined under a scanning electron microscope. Marked degeneration of the organ of Corti in all turns and dislocated and encapsulated tectorial membrane were found in the cochlea. Marked decrease in number of the sensory cells in the utricular and saccular maculae and total loss of sensory hair bundles in the ampullary cristae of the semicircular canals were seen in the vestibule. These findings strongly suggest that the cause of sudden deafness in this case might be viral. The usefulness of scanning electron microscopy in human temporal bone pathology is stressed.

(SEM) Scanning electron micrographs disclosed inner ear pathologies typically found in cases of known viral labyrinthitis as well as described below. The SEM method is useful to obtain a three dimensional view of changes and to visualize more highly magnified pictures than a light microscope. We will be able to produce

CASE HISTORY

Though sudden deafness is not a rare occurrence in the otolaryngologic clinic, studies on inner ear pathology are still scanty. Inner ear findings in cases of sudden deafness of unknown etiology showed changes similar to those found in known viral labyrinthitis (Beal et al., 1967; Schuknecht et al., 1973) and, though fewer in number, in vascular disorder (Gussen, 1976) and in probable cochlear neuronitis (Ishii, 1977). The temporal bone studies in the above reports were done using celloidin sections under the light microscope.

The authors of this paper were able to obtain a pair of temporal bones from a case of bilateral sudden deafness which occurred 1 year before the patient's death. The right ear is now being processed for study using the conventional celloidin method. The left ear was processed for scanning electron microscopy

A 56-year old female complained of stiffness, fever, swelling and congestion of the skin at the root of her nose, since April 1976. On the morning of September 13, 1976 she noticed loud tinnitus in her left ear and loss soon afterwards. On the next day she experienced the same hearing disorders in the right ear and became totally deaf on both sides. The patient did not have acute respiratory infections at that time. She felt unsteady when walking after these episodes. As she had recurrent swelling, redness and pain in the auncles since October, she was admitted to a university hospital in December 1976.

Upon admission she showed a saddle-shaped deformity, deformed auncles with redness and pain on both sides and congestion of the epiglottis. The following laboratory data were noted: RBC, 306×10^4 , WBC, 20,100 per mm³.



Fig. 1 Surface view of the cochlear apical turn. Encapsulated tectorial membrane is lying for the most part on the outer sulcus (arrows). In the basal part the tectorial membrane is incorporated onto the organ of Corti (C). No

outer hair cells are visible. The area in the box is shown in Fig. 2 under higher magnification. R: Reissner's membrane, L: limbus spiralis.

R 1° 150 mm, CRP, 5+, RA, +, Wa-R, -, serum protein, 7.6 g/dl, A/G, 0.64, Ig-A, 1 mg/dl, Ig-G 2800 mg/dl.

Histological study of biopsied auricular tissue showed degeneration of cartilaginous tissue and marked infiltration of inflammatory cells and was diagnosed as chondritis. No signs of ear disease were observed at otoscopy. Audiometry showed complete hearing loss in both ears. Caloric test with 20 ml ice water irrigation did not elicit any responses. Despite administration of steroids, immunosuppressant (azathioprine) and antibiotics, the patient took a gradual downward course and died of gastrointestinal hemorrhage on 1 October, 1977. During the entire course of illness, no treatment with aminoglycoside antibiotics or irradiation therapy was used. Autopsy was done 3 hours after death.

METHODS

Both temporal bones were removed during autopsy and the left ear was immediately prepared for SEM examination by introducing

2% glutaraldehyde in 1/15 M Sorensen buffer (pH 7.4) several times through the round and oval windows. The bone was further fixed in the same fixative for about 24 hours and was subsequently kept in the phosphate buffer.

After drilling down the bony capsule the inner ear was dissected in the buffer. The cochlea was cut into half turn lengths along the plane running from the modiolus to the border between the oval and round windows. Dissected sections were then treated with Murakami's electron conductive fixation method using 2% tannic acid and 1% OsO_4 (Murakami, 1974). Specimens were dehydrated in graded ethanols, transferred into amyl acetate and dried in a critical point dryer using liquid carbon dioxide. Dried specimens were glued on a brass stub, coated with gold/palladium by an ion sputter and observed under a scanning electron microscope (JSM35).

After observation by SEM, some selected parts of the same specimen were dissected and embedded in Epon. One micron sections were then cut using a glass knife and these were observed under a light microscope.



Fig 2 Encapsulated and incorporated tectal membrane (upper turn). Look through a break in the encapsulating thin cell layer the tectal membrane is seen to its fibrillar structure (T). Degenerated inner hair cells (arrows) and distorted inner pillar cells (P) are visible. S, inner sulcus cell.

FINDINGS

Middle ear structures were not preserved in the specimens because they were removed during autopsy. The otic capsule seemed to be more sclerotic than in usual specimens. During inner ear dissection no fibrous or bony tissue proliferations or hemorrhage were observed. Also no anomalies were found in the cochlea or vestibule. As specimens were prepared so as to expose the organ of Corti to the best possible advantage the spiral ligament was partly cut and removed and Reissner's membrane was cut leaving only a small part at the insertion to the spiral limbus. It was not ascertained whether the degree of expansion of Reissner's membrane or of the membranous wall of the vestibule was normal or not.

Cochlea

The most noticeable pathological changes in the cochlea were a rolled up and encapsulated

tectal membrane and a marked degeneration of the organ of Corti.

The tectal membrane was visible in a turns except most of the upper basal turn and the beginning of the lower basal turn. For the most part the tectal membrane was lying in the inner sulcus and at places on the organ of Corti, Reissner's membrane and on the spiral limbus (Fig 1). The tectal membrane itself was completely covered by a layer of thin flat cells. This covering layer was composed of different types of cells corresponding to the site upon which they rested. Where the tectal membrane was lying on the organ of Corti or the inner sulcus the covering cells had large polygonal surfaces (Fig 2). When the tectal membrane rested on Reissner's membrane the covering cells were smaller, round and had many microvilli (Fig 3B). The encapsulated tectal membrane still seemed to keep its fibrillar structure as was observed through

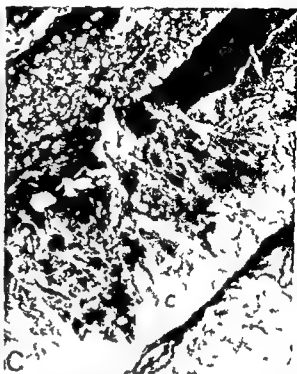


Fig 1 Adhesion of encapsulated tectorial membrane to the organ of Corti (A, B) and destruction of the organ of Corti after ripping off incorporated tectorial membrane (C). The covering cells have round shapes and many

microvilli in B (lower basal turn). In (C) the tectorial membrane was partially incorporated on the organ of Corti (C). Only inner hair cells are visible (arrow). R: Reissner's membrane. L: limbus spiralis.

small breaks in the monolayer cell covering (Fig 2).

Both the thin cell covering and the tectorial membrane itself were adhered to and incorporated into whichever underlying structure they rested upon (Figs 1, 2), for when parts were detached during preparation the underlying part showed a breakdown in structure as if the tectorial membrane had been ripped off (Fig 3C).

In normal cases the surface of the limbus spiralis is usually covered by a thin layer of the tectorial membrane and its cellular structure is hardly visible. In this case however the surface was not covered at all by the tectorial membrane. Being hidden by round globular projections a cellular structure with polygonal surfaces was visible, probably being interden- tal cells (Fig 4A).

Degeneration of the organ of Corti was

marked in all turns, being most drastic towards the basal turn. All the outer hair cells had disappeared and in turn were replaced by a proliferation of surrounding cells. Surfaces of the inner pillar cells were recognizable only in the apical turn. In lower turns the inner pillar cells were replaced by polygonal surfaces of what are probably the changed shape of Deiters cells. In the lower basal turn the mound structure of the organ of Corti had degenerated in places into totally flat parts. In the cut sections Corti's tunnel and Nuel's space were hardly recognizable in all turns because of proliferation of cellular structure (Fig 4B, C).

Inner hair cells with degenerated sensory hairs were seen in every turn except at the basal end of the cochlear duct where they had totally disappeared. The sensory hairs lost their normal arrangement and regular stepwise gradation by height (Fig 5). Abno-

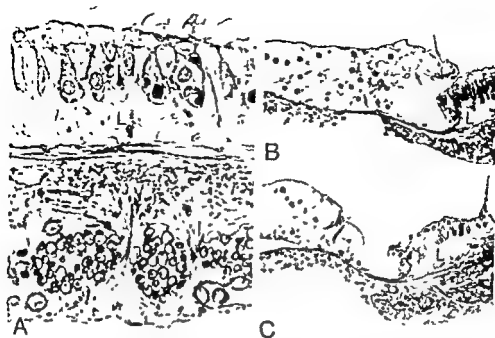
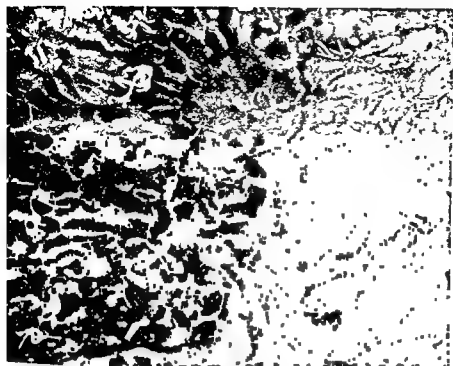


Fig 4 Cut sections of the spiral limbus and the organ of Corti (A) Surface of the spiral limbus (L) is covered by interstitial cells. Myelinated nerve fibers are fairly well preserved (upper basal turn) (B) The tectorial membrane

is incorporated into the organ of Corti (arrow) (lower middle turn) Both the tunnel of Corti and Nuel are occupied by proliferation of cellular structure in B and (lower middle turn)



Fig 5 Degenerated inner hair cells (apical turn) Regular stepwise arrangement of sensory hairs is lost. Large hair-like protrusions are abundant



6 Atrophied saccular macula. Sensory cells markedly decreased in number and are replaced by flat cells with large surfaces

ly large hair-like projections were frequently observed

During fixation of the specimen with OsO_4 , the osseous spiral lamina was examined under light microscope. Here the network of eliminated fibers seemed to be fairly well preserved except in the basal turn. A slight decrease in number and patchy degeneration were seen in the lower basal turn. Myelinated nerve fibers were further ascertained in the cuttings of the same specimens (Fig. 4A).

The spiral ligament showed a normal thickness and texture. The stria vascularis was cut during preparation and was not preserved in the observed specimens. Reissner's membrane also showed its usual appearance, except for the lower middle and lower basal parts where the tectorial membrane had fallen off and adhered to it.

Utricle

During dissection of the specimen, a decrease in number of otoconia was seen in the utricle

and the saccule. The gelatinous layer of the otolithic membrane also seemed to be thinner than usual.

Observation of the saccular macula showed a great decrease in the number of sensory hair bundles over the entire surface (Fig. 6). The remaining sensory hair bundles showed a decrease in the number of sensory hairs and the hairs themselves showed pronounced abnormalities in arrangement and shape (Fig. 7). The decrease in the number of sensory cells was not so marked on the utricular macula as in the saccule. The central part of the utricular macula, however, was entirely devoid of sensory hair bundles and in their place were found flat cells with large surfaces. While all the sensory hair bundles on the saccular macula were degenerated, on the utricular macula many normal looking hair bundles still remained.

The ampullary cristae of the anterior and lateral semicircular canals showed a complete loss of sensory hair bundles both



Fig. 7
Regl.

central and peripheral parts (Fig. 8). The posterior canal crista was not examined as it had been damaged during dissection. Prior observation in every vestibular endorgan under a dissection microscope showed that the myelinated nerve fibers were fairly well preserved.

COMMENTS

The pathological manifestations which we observed—marked atrophy of the organ of Corti, encapsulated and dislocated tectorial membrane with no noticeable decrease in the myelinated nerve fibers—are all recognized as histological characteristics of viral labyrinthitis (Lindsay 1967). This same sort of encapsulated tectorial membrane has also been reported in genetically determined deafness (Gussen 1968) and also in congenital

deafness of unknown origin (Cohn et al 1968). For our patient there was no family history of deafness, thus sudden deafness in this case may well be the result of viral labyrinthitis.

Viral labyrinthitis and blood circulation disorders have been postulated as the two most probable causes of sudden deafness in which the etiology is not so obvious (Schuknecht et al 1973). Our findings give further support in favor of the theory of viral labyrinthitis as a cause of sudden deafness.

The exact cause of the relapsing polychondritis has not yet been determined. This disease is known not infrequently to accompany sensorineural deafness. It has been suggested that vascular impairments (presumably arteritis of the internal auditory artery) are the causative factors of this specific



Fig 2 Lateral crista ampullaris viewed from the utricular side. The surface of the sensory cell area is completely

devoid of sensory hair bundles. The surface is covered by flat cells and many globular protrusions.

and vestibular disorders (Cody et al., 1971). Among 40 relapsing polychondritis cases reported by Cody et al (1971), profound deafness in 4 cases progressed rapidly within 48 hours. Medical histories of these suddenly occurred deafness cases showed a great resemblance to the case we have reported in this paper. The authors are of the opinion that the rapidly progressing deafness cases occurring during the course of relapsing polychondritis might be caused by viral labyrinthitis. However, many more pathological studies on the temporal bone in relapsing polychondritis cases are necessary for further clarification of the etiology of this deafness.

Using a transmission electron microscope (TEM), Nadol (1977) observed the inner ear in a case of profound deafness which was probably caused by viral labyrinthitis. An encapsulated tectorial membrane was found in parts of the cochlear duct. Because he noticed a fusion of

the tectorial membrane and the connective tissue inside the spiral limbus through a dehiscence of the epithelial layer of the vestibular lip, Nadol mentioned the possibility that the encapsulated substance might be prolapsed connective tissue of the limbus spiralis. In our own case, the encapsulated tectorial membrane was found even on Reissner's membrane without any connection to the limbus spiralis. The tectorial membrane observed through breaks in the covering cell layer showed the characteristic fibrillar structure usual to the tectorial membrane. These findings seem not to agree with Nadol's assumption, as mentioned.

Because of morphological similarities the origin of the layer of cells covering the tectorial membrane seemed to be either the cells of the inner sulcus or the cells of the epithelial layer of Reissner's membrane, depending on where the tectorial membrane rested.

labyrinthitis occurred, the tectorial membrane was probably detached from the limbus spiralis. Resultant damage to the epithelial cells left marked degeneration of the sensory cells on one side and, on the other hand, caused proliferation of inner sulcus cells and cells of Reissner's membrane through the breakdown of tight junctions. These proliferated cells then covered the overlying detached tectorial membrane and then later incorporated themselves with the underlying tissue in the healing stage.

The vestibular degeneration which we observed is a rather unique feature according to the findings reported so far on known viral labyrinthitis. Degenerative changes in the vestibule in viral labyrinthitis are reported to be mild or confined only to the saccule, showing Scheibe type cochleo-saccular degeneration. Vestibular organs of the sudden deafness cases reported by Beal et al (1967) and Schuknecht et al (1973) also showed findings similar to those in cases of viral labyrinthitis. As far as can be found from the reports, temporal bones in measles cases (Lindsay et al, 1954; Schuknecht, 1974) were the only ones which showed mild degeneration in the pars superior of the inner ear. However, in our case, marked degeneration of the vestibule was found in the cristae ampullares, the saccular macula and utricular macula, in this order of severity. The type of virus responsible in the present case could not be ascertained. Therefore it is not certain whether the causative virus had affinity to the superior portion of the inner ear or whether the destructive process was more marked than the other cases so far reported.

Scanning electron microscopy as applied in this study seems to be one of the most promising methods in human temporal bone histopathology. The lower magnifications enable one to survey the overall structure of the cochlear turns. Higher magnifications enable one to examine changes at cell level. The parts of the inner ear which were examined do not seem to be largely affected by post mortem autolysis.

After observation by SEM the same specimen can be cut by embedding it either in Epon or in paraffin and then further examined by light microscope or TEM. The stria vasculans which regrettably had to be omitted in the present investigation, can be examined in the cut specimen by light microscope and TEM if the tissues containing the stria vasculans are preserved during processing. Nerve fibres in the osseous spiral lamina can be checked either by light microscope during specimen preparation, or after SEM observation by light microscope or TEM. The drawbacks of the SEM method are difficulty in assessing the position of Reissner's membrane and analysing the spiral ganglion cells. When examining a pair of temporal bones in which the same pathologies are expected, the authors recommend processing one ear for SEM and the other ear for the conventional celloidin method.

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ZUSAMMENFASSUNG

Das linke Ohr einer 57-jährigen Kranken mit Reibel-Polychondritis, die den plötzlichen Hörsturz bekam.

Die histopathologischen Untersuchungen des Innenohrs zeigten eine schwere Degeneration der Ampullen des Bogengangs, der Cristae ampullares, der Saccular macula und der Utricular macula, in dieser Reihenfolge der Schwere. Der Typus des Virus, das für die Erkrankung verantwortlich ist, konnte nicht festgestellt werden. Daher ist es nicht sicher, ob das verursachende Virus eine Affinität zum oberen Teil des Innenohrs hat oder ob der zerstörerische Prozess stärker ausgeprägt war als in anderen Fällen. Die Scanning-Elektronen-Mikroskopie (SEM) scheint eine der vielversprechendsten Methoden in der menschlichen Temporal-Knochen-Histopathologie zu sein. Die niedrigeren Vergrößerungen ermöglichen es, die Gesamtstruktur der Cochlea-Touren zu untersuchen. Höhere Vergrößerungen ermöglichen es, Veränderungen auf Zellebene zu untersuchen. Die Teile des Innenohrs, die untersucht wurden, scheinen nicht stark von post mortem Autolyse betroffen zu sein.

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THE EFFECTS OF EXPERIMENTALLY PRODUCED MIDDLE EAR LESIONS ON TYMPANOMETRY IN CATS

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Abstract Tympanometry was performed before and after producing specific lesions in the middle ears of cats. The lesions selected for study included stapes fixation, ossicular discontinuity and scarred tympanic membranes. Stapes fixation resulted in marked increases in middle ear impedance easily detected with tympanometry. Ossicular discontinuity resulted in complex tympanometric shapes which were easily accounted for by simple interactions between acoustic resistance and reactance. The complex shapes that occurred in normal and abnormal ears with pressure changing from negative to positive resulted from more complicated interactions. Large surgical lesions in the posterior superior quadrant of the drum were quite visible at otoscopy but could not be detected tympanometrically one month after surgery.

Since Terkildsen & Thomsen (1959) originally described the technique, tympanometry has become a widely accepted clinical procedure for the detection of middle ear abnormalities. Their original procedure involved measurements of the effects of ear canal air pressure changes on the sound pressure level (SPL) of a tone introduced into the auditory meatus. These SPL changes reflect changes in the acoustic input impedance of the middle ear resulting from ear canal pressures that are either positive or negative with respect to atmosphere. The technique has been modified by many investigators so that the term tympanometry now refers to a class of observations in which any of a variety of acoustic quantities is measured as a function of ear canal pressure. Consequently, the resulting plot (tympano-

gram) can take a variety of forms depending on the quantity selected for study.

Regardless of which acoustic quantity is measured, normal tympanograms usually take an inverted 'V' shape indicating maximum admittance (or minimum impedance) near ambient ear canal pressure. Abnormal tympanograms can be grossly categorized as reflecting abnormally high impedance systems or abnormally low impedance systems. High impedance systems most commonly result from such pathologies as serous otitis media and ossicular fixation. These conditions often manifest themselves in a flattening of the normally peaked tympanogram. Although Zwislocki (1957) reported substantial differences in middle ear impedance between normal and otosclerotic subjects, Jerger et al (1974) reported that one cannot readily differentiate the otosclerotic tympanogram from the normal tympanogram (p 166). This apparent discrepancy can perhaps be resolved by studying the tympanometric effects of experimentally produced stapes fixation in cats.

Low impedance systems are characterized by tympanograms with very high peaks and/or multiple peaks. A recent report by Vanhuyse et al (1975) suggested an analytic approach which has revealed some relationships that ac-

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count for multiple-peaked tympanograms. They postulated that interactions among three quantities—acoustic resistance, acoustic reactance and ear canal air pressure—might account for the multiple-peaked tympanograms that have been observed in normal ears (Albert & Jerger, 1974, Colletti, 1975, 1976), ossicular disruption (Liden, Harford, and Hall 1974), and eardrum abnormalities (Feldman 1974). Liden et al (1977) and Margolis & Popelka (1977) studied tympanograms from normal and pathological human ears and basically confirmed the rules outlined by Vanhuyse et al that govern the occurrence of double peaked tympanograms. These rules are most clearly described by the graphic presentation provided by Vanhuyse et al and may be summarized as follows. Double-peaked tympanograms occur when 1) acoustic reactance is stiffness-controlled and less than acoustic resistance near ambient ear canal pressure and greater than acoustic resistance at high pressures, and 2) when acoustic reactance is mass-controlled near ambient pressures and stiffness controlled at high pressures. When double-peaks occur in acoustic conductance, susceptance and impedance tympanograms, the acoustic resistance and reactance tympanograms usually have much simpler shapes.

Margolis & Popelka (1977) suggested that double peaked tympanograms at 660 Hz may occur in both normal and pathological ears. Double peaked tympanograms from stiffness-controlled ears can be accounted for on the basis of normal interactions among tympanometric quantities, while those that occur in mass controlled ears probably result from some past or present pathology. The distinction between mass-controlled and stiffness-controlled reactance at 660 Hz may provide a simple method for identifying the double-peaked tympanograms that result from pathology. This distinction can easily be made on the basis of the B_a tympanogram. If the minimum in the center of the notch is the lowest point on the tympanogram, the ear is mass controlled

Although there is a substantial literature concerned with tympanometric results in pathological ears, the effects of specific middle ear lesions on tympanometry are not well understood. This confusion derives in large part from poor control over the nature and extent of the lesions in clinical populations of human subjects. We undertook the present experiments to 1) study the effects of specific, experimentally-produced middle ear lesions on tympanometry in cats, and 2) evaluate the Vanhuyse et al predictions concerning the nature of W-shaped tympanograms. The lesions selected for study included stapes fixation and ossicular discontinuity (Experiment I) and healed tympanic membrane perforation (Experiment II).

EXPERIMENT I

Methods and Materials

Tympanometric measurements were performed by simultaneously monitoring the acoustic conductance (G_a) and acoustic susceptance (B_a) outputs of an acoustic admittance meter (Grason Stadler 1720B) with a two-channel strip-chart recorder (Gould 220). A third pen was used as a manually-triggered event marker that provided estimates of ear canal air pressure. The event marker was triggered by the experimenter in 50 mmH₂O intervals during each tympanometric record. Since air pressure is a linear function of time with this instrument, reasonable estimates of ear canal pressure can be obtained in this manner. For each experimental condition, tympanograms were recorded with two probe frequencies (220 and 660 Hz) with both increasing (−400 to +400 mmH₂O) and decreasing ear canal air pressure. All data were corrected to the plane of the tympanic membrane by the MAX/MIN procedure (see Margolis & Smith, 1977). Acoustic admittance data (B_a and G_a) are expressed in acoustic mmhos, acoustic impedance data (X_a and R_a) are expressed in acoustic ohms. One acoustic ohm is defined as $\text{m}^2 \times 10^3 / \text{Pa} \cdot \text{sec}$, one acoustic ohm is defined as $\mu\text{Pa} \cdot \text{sec} / \text{m}^2$.

Table I^a

		220 Hz					660 Hz				
		G_a	B_a	R_a	$-jX_a$	Z_a	G_a	B_a	R_a	$-jX_a$	Z_a
Normal (A) (before drilling) ($N=13$)	\bar{X}	0.05	0.49	214	2.147	2.161	0.64	1.47	245	559	625
	S D	0.30	0.08	158	359	366	0.31	0.37	132	108	94
Normal (B) (after drilling) ($N=8$)	\bar{X}	0.06	0.50	223	2.031	2.046	0.49	1.47	205	624	660
	S D	0.04	0.10	108	380	380	0.21	0.23	111	170	123
Stapes fixation ($N=6$)	\bar{X}	0.05	0.25	713	3.929	3.999	0.14	0.83	230	1.213	1.139
	S D	0.02	0.03	105	671	649	0.08	0.18	141	.780	.793
Stapedectomy ($N=8$)	\bar{X}	0.46	1.87	106	722	734	1.55	1.97	743	301	494
	S D	0.51	1.13	111	475	470	1.83	1.31	256	213	700
Incudo-stapedectomy ($N=6$)	\bar{X}	1.50	1.97	223	568	631	1.58	0.89	375	768	517
	S D	1.60	1.18	161	470	465	0.98	1.02	238	750	148

^a G_a and B_a data are in acoustic mmhos. R_a , X_a and Z_a data are in acoustic ohms.

Experimental subjects included 13 cats, otoscopically screened for obvious middle ear pathology. After the cats were sedated with Nembutal (35 mg/kg intraperitoneal) the pinna and all but the medial 1.0 cm of the ear canal were removed. The middle ear was entered through a 2.5 mm burr hole placed 4 mm posterior-superior to the opening of the bony ear.

With the aid of a binocular microscope following middle ear manipulations were

First the stapes was immobilized with methyl methacrylate glue (Eastman 810). Then the stapes was removed. Finally, the incus was removed.

Tympanograms were recorded after each of these manipulations with the burr hole sealed with a plug made from dental cement. Experimental conditions included the following: There were two baseline conditions, one before and one after drilling into the middle ear. This allowed the determination of the potential traumatic effect of drilling on tympanometric results. There were three experimental conditions representing each of the experimentally-produced lesions: stapes fixation, stapedectomy, and incudostapedectomy. Tympanograms, recorded in acoustic conductance G_a , and susceptance, B_a , were converted to acoustic resistance, R_a , reactance X_a and impedance, Z_a , by the relations derived by Van Camp (1977) and Margolis & Popelka (1977). Although measurements were made from 13

cats, surgical complications required the elimination of several animals from the experiment. The most common problems were tympanic membrane rupture and excessive glue in the middle ear. Agreement between tympanograms recorded before and after drilling was required for inclusion of the experimental data. Table I presents the pre and post-drilling results (Baseline A and B) indicating excellent agreement for those subjects retained in the experiment.

Results and Discussion

Direction of pressure change

Fig. 1 presents representative results from one cat illustrating the effects of direction of pressure change. Similar relations were evident in the data from each animal. These tympanograms were recorded with a 660 Hz probe before drilling into the middle ear. In the decreasing (positive to negative pressure direction) G_a and B_a tympanograms assume the normal, single peaked tympanometric shape. R_a and X_a show the relations predicted by Vanhuyse et al. (1975) and confirmed in human subjects by Liden et al. (1977) and Margolis & Popelka (1977). Specifically, acoustic reactance is greater (in absolute value) than resistance for all ear canal pressures and resistance assumes a grossly asymmetrical shape. In the increasing pressure direction G_a and B_a tympanograms are substantially differ-

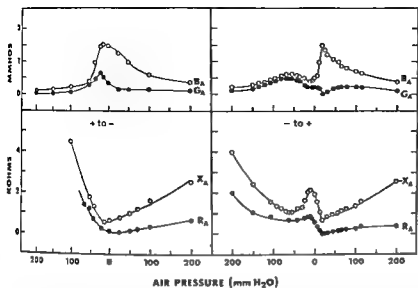


Fig 1 The effects of direction of pressure change on tympanograms from one cat. The data in the left panels were recorded with pressure changing from positive to negative with respect to atmosphere. The data on the right were recorded in the opposite direction. The top panels

present acoustic susceptance (B_a) and conductance (G_a). The bottom panels are acoustic reactance ($-X_a$) and resistance (R_a) computed from the B_a and G_a data. All data are corrected to the plane of the tympanic membrane by the MAX/MIN procedure (see Margolis & Smith 1977).

At about 20 mmH₂O acoustic conductance is very near zero. Acoustic susceptance has a double peak with the larger peak corresponding to the minimum in the G_a tympanogram. The resistance and reactance tympanograms take much more complicated forms than occurred with the other pressure direction. Both are asymmetrical and double-peaked. Several investigators have reported differences in tympanograms corresponding to different pressure directions. The position of the tympanometric peak was found to be dependent upon pressure direction by Woodford et al (1976).

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ams & Feldman (1976) found a higher occurrence of double peaked tympanograms when pressure changed from negative to positive than for the reverse direction. These findings are similar to the present study. However the double peaked B_a tympanogram in Fig 1 appears to be unrelated to those predicted by Vanhuysse et al (1975) and reported by Liden

et al (1977) and Margolis & Popelka (1977). In those reports double peaked G_a and B_a tympanograms never resulted from double peaked R_a and X_a tympanograms like those shown in Fig 1. Rather, much simpler tympanometric patterns in R_a and X_a resulted. It appears then, that the double peaked tympanograms that occur with the increasing pressure direction result from a different kind of interaction between resistance and reactance than those that were predicted by Vanhuysse et al. In the following discussion of the effects of middle ear lesions, only tympanograms recorded with the decreasing (positive to negative) direction will be considered.

Normal ears

Figs 2 and 3 present acoustic conductance and susceptance tympanograms from one representative animal for two experimental conditions—stapes fixation and ossicular continuity. Acoustic resistance and reactance computed from the measured

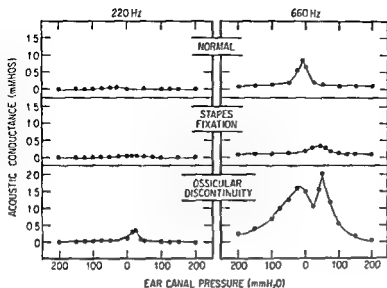


Fig 2 Acoustic conductance tympanograms from one cat at two probe frequencies (220 and 660 Hz) under three experimental conditions. Normal after drilling into the bulla with the drill hole seal; stapes fixation after cementing the stapes footplate; ossicular discontinuity after removal of the stapes. All data are corrected to the plane of the tympanic membrane.

and susceptance values are presented in Figs 4 and 5 for two probe frequencies, 220 and 660 Hz, and two experimental conditions—the normal control condition and ossicular discontinuity. Averaged data for all conditions, probe frequencies, and acoustic quantities are summarized in Table 1.

Tympanograms recorded from normal cats are similar to those obtained with human subjects. Conductance and susceptance tympanograms (Figs 2 and 3) have single

peaks located near ambient ear canal pressure with the exception of G_a at 220 Hz which is similar to human infant data (Margolis & Popelka, 1975), does not have a clearly defined peak. The relations between acoustic conductance and reactance (Figs 4 and 5) are similar to those described for normal human subjects (Vanhuyse et al 1975, Liden et al 1977, Margolis & Popelka, 1977). Specific acoustic reactance is stiffness controlled and is greater (in absolute value) than acoustic

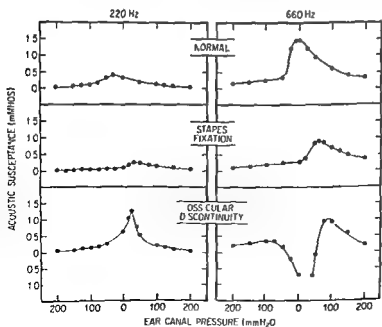


Fig 3 Acoustic susceptance tympanograms from one cat. See caption to Fig 2.

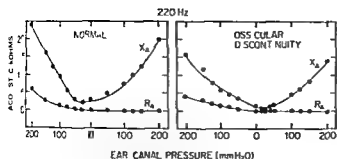


Fig 4 Acoustic resistance (R_a) and reactance ($-X_a$) tympanograms computed from the 220 Hz data in Figs 2 and 3 for the normal condition and after removal of the stapes

istance for all ear canal pressures at both probe frequencies. Furthermore, acoustic reactance is nearly symmetrical about ambient pressure while resistance takes a grossly asymmetrical shape with lower values for positive than for negative pressures. These relations are associated with single-peaked B_a and G_a tympanograms in humans and, as these data suggest in cats as well. Static acoustic impedance (see Table I and Fig 6) is somewhat higher than for adult human subjects (Margolis & Smith, 1977) but similar to 2-4 month human infants (Margolis & Popelka 1975).

Stapes Fixation

Figs 2 and 3 present tympanometric results from one cat illustrating the effects of stapes fixation on acoustic susceptance and conductance tympanograms. In all cases there was a marked decrease in the height of the tympanometric peak except for the 220 Hz G_a tympanogram which in normal animals did not have a clearly defined peak. These changes were associated with increases in both acoustic resistance and acoustic re-

actance, although at 660 Hz, the resistive change was small (Table I). As Zwislöcki (1957) pointed out, stapes fixation produces a marked increase in middle ear impedance. The Jerger et al (1974) contention that normal subjects and otosclerotics cannot be differentiated on the basis of tympanometry may be related to the following considerations. First, the effects of otosclerosis on the input impedance of the human middle ear may be fundamentally different from the effects of our experimentally-produced stapes fixation in cats. Second, normal variability among human subjects is probably greater than among our carefully selected animal subjects, making departures from normality more difficult to detect in humans. Third, the quantification of tympanometric data in arbitrary units and the classification of tympanograms into a few poorly defined categories may obscure potential differences that could be detected by more rigorous measurement procedures. On visual observation the tympanograms shown in Figs 2 and 3 for the stapes fixation condition are not clearly different from normal. However, when the data are converted to estimate the static

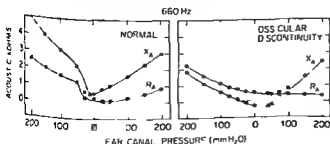


Fig 5 Acoustic resistance (R_a) and reactance ($-X_a$) tympanograms computed from the 660 Hz data in Figs 2 and 3 for the normal condition and after removal of the stapes

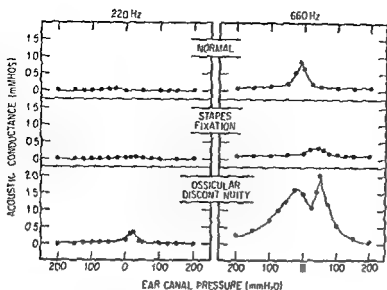


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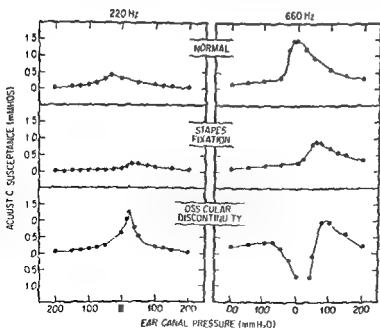


Fig 3 Acoustic susceptance tympanograms from one cat. See caption to Fig 2.

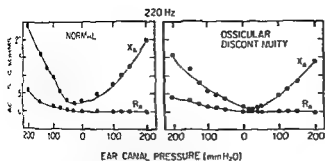


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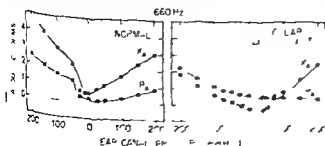


Fig 5 Acoustic resistance (R_a) and reactance ($-X_a$) tympanograms computed from the 660 Hz data in Figs 2 and 3 for the normal condition and after removal of the stapes

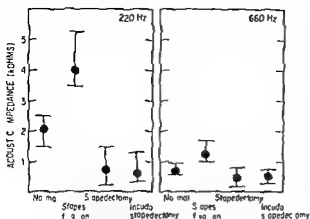


Fig 6 Mean and range static acoustic impedance (corrected to the plane of the tympanic membrane) for all experimental conditions at two probe frequencies (220 and 660 Hz)

acoustic impedance of the middle ear, the two groups—normal and stapes fixation—separate into two distinctly different distributions (see Fig 6)

Ossicular discontinuity

Representative results from one animal after removal of the stapes are illustrated in Figs 2-5. At 220 Hz, ossicular discontinuity resulted in single-peaked B_a and G_a tympanograms, although when compared with the normal control condition the amplitudes of the peaks were increased (Figs 2 and 3). Fig 4 illustrates that the relationship between resistance and reactance is similar to the normal condition. That is, reactance is stiffness-controlled and always greater, in absolute value, than resistance. At 660 Hz, susceptance and conductance tympanograms have double peaks (Figs 2 and 3). In Fig 5 it is evident that the normal relation between resistance and reactance is altered. In the stapedectomized subject, reactance is mass controlled near ambient ear canal pressure and stiffness-controlled at high positive and negative pressures. The data obtained after removal of the incus were qualitatively similar to the stapedectomy condition. The B_a and G_a tympanograms tended to be more dramatically notched and the reactance tympanogram was more displaced toward positive values. In

general, the results from cats with ossicular discontinuity are similar to the data we reported from a human subject with a suspected discontinuity (Margolis & Popel 1977, figures 9 and 10). Both the human and feline data support the Vanhuyse et al predictions regarding interactions among R_a and that account for double peaked tympanograms. Specifically, double peaked and G_a tympanograms occur when reactance is less than resistance and/or mass control near ambient pressure, and reactance stiffness controlled and greater than resistance at high ear canal pressures.

Summary and Conclusion

Mean static acoustic impedance data for experimental conditions are summarized in Fig 6. Stapes fixation resulted in a marked increase in acoustic impedance at both probe frequencies. Ossicular discontinuity (stapedectomy and incudo-stapedectomy) resulted in a clear decrease in acoustic impedance at 220 Hz. At 660 Hz the effect of ossicular discontinuity on static impedance was not as dramatic, even though all of the stapedectomized ears and all incudo-stapedectomized ears were mass controlled. The lack of separation between the normal and the ossicular discontinuity groups (Fig 6) is due to the fact that static impedance values are insensitive to the sign of reactance. That is, a mass controlled ear may have the same static impedance as a normal stiffness-controlled ear. It is necessary, therefore, to analyse the components of acoustic impedance if high frequency probe tones are to be clinically useful.

The interactions among resistance and reactance, predicted by Vanhuyse et al (1977) to account for multiple peaked B_a and G_a tympanograms have been confirmed in human subjects by Lidén et al (1977) and Margolis & Popelka (1977), and now in mass controlled middle ear lesions produced in experimental animals. Double peaked tympanograms that occur in normal cats with

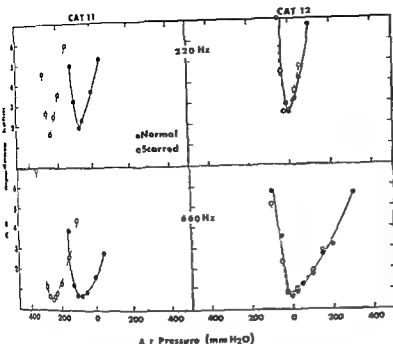


Fig 7 Acoustic impedance tympanograms ($Z_{e/f}$) from 2 cats at two probe frequencies (220 and 660 Hz). One ear of each cat was perforated one month before these measurements were made. The data are corrected to the plane of the tympanic membrane.

660 Hz probe and with pressure changing from negative to positive are an exception to the Vanhuysse et al predictions. These tympanograms are characterized by complex distance and reactance patterns that represent a more complicated set of interactions than those that seem to be associated with ossicular disruption and eardrum abnormalities. The effect of pressure direction on tympanometric shape in human subjects requires further study. If effects similar to those depicted in Fig 1 can be demonstrated in human subjects, more attention should be paid to the direction of pressure change in clinical procedures.

EXPERIMENT II

Methods and Material

Tympanograms were recorded from the operated ear and the unoperated control ear of 2 cats 1 month after perforation of the tympanic membrane. In one ear of each cat a 2 mm perforation was produced with a very fine knife in the posterior-superior quadrant of the eardrum. This region has been shown to vibrate maximally to acoustic stimulation.

(Khanna & Tonndorf 1971). Thus we reasoned that if a scar resulting from a surgical incision would influence tympanometry, its effect would be maximal in this location. Measurement procedures were identical with those of Experiment I.

Results and Discussion

Although the scars were easily visible at otoscopy, there was no apparent effect on tympanometric results. Fig 7 presents acoustic impedance tympanograms from both ears of both experimental animals at two probe frequencies. Cat 11 had a substantial amount of negative pressure in the operated ear but 7% measures were not appreciably different. Cat 12 had virtually identical tympanograms from the two ears.

Conclusion

The data of Experiment II suggest that scars resulting from surgical incisions, even when carefully placed, do not influence tympanometric results. This is in contrast to the findings of Khanna & Tonndorf (1971) who reported that scars in the posterior-superior quadrant of the eardrum influenced tympanometry.

typanometry. Results from human subjects have demonstrated remarkable effects of tympanic membrane abnormalities on tympanometric data (Feldman, 1974; Margolis & Popelka, 1977). These eardrum abnormalities represent gross changes in the mechano-acoustic properties of the eardrum that result from anatomical changes that characterize healed spontaneous perforations. The present data do not contradict the well-documented effects of scarred eardrums, but demonstrate that the healing that occurs after surgical incisions does not leave the eardrum in a condition that is mechanically different from the normal ear.

ZUSAMMENFASSUNG

Tympanometrie wurde vor und nach Erzeugung von spezifischen Schädigungen des Kaltenmittelohrs durchgeführt. Die für diese Arbeit ausgewählten Schädigungen bestanden aus Steigbügelversteifung, Unterbrechung der Hörknöchelchen und narbigem Trommelfell. Die Steigbügelversteifung verursachte deutliche Erhöhungen in der Mittelohrimpedanz, die durch Tympanometrie leicht entdeckt wurde. Unterbrechung der Hörknöchelchen hatte komplizierte tympanometrische Formen zur Folge, die durch einfache Wechselwirkungen zwischen akustischem Widerstand und Reaktanz leicht erklärbar waren. Die komplizierten Formen, die in normalen und geschädigten Ohren vorkamen, als der Luftdruck von negativ auf positiv gebracht wurde, ergaben sich von komplizierteren Wechselwirkungen. Große chirurgische Einschnitte im hinteren, oberen Quadrant des Trommelfells waren mittels Otoskopie ganz sichtbar, konnten aber einen Monat nach dem Einschnitt durch Tympanometrie nicht entdeckt werden.

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NASAL CANCER ASSOCIATED WITH OCCUPATIONAL EXPOSURE TO ORGANIC DUST

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Abstract In order to study the association of carcinoma of the nose and paranasal sinuses with occupational exposure to dust the cancer registry of the National Board of Health and Welfare was consulted as information source. The material was divided into two groups: the first consisting of consecutive cases of adenocarcinoma from 1961 until 1971 and the second of cases of squamous cell and poorly differentiated carcinoma from 1965 until 1971. The

predominant occupational group among men with adenocarcinoma was joiners who comprised 19 cases (53%). At least 12 of these had been cabinet makers. The period of exposure to wood dust among the joiners was known in 9 cases and was estimated in most of the cases. The duration of exposure was not less than 25 years in any case. Most joiners had been working with different species of hard wood. There was no predominant occupational group among men with squamous cell and poorly differentiated carcinoma although the occupations recorded were in many cases associated with a working environment containing various kinds of organic dust. Among the females there were a number of cases who had occupations associated with organic dust such as textile work, leather and flour handling. The investigation shows that the bulk of males with adenocarcinoma of the nose and paranasal sinuses had been exposed to wood dust.

sen, 1975), France (Gignoux & Bernard, 1969, Gignoux et al., 1971), Holland (Delemarre & Themans, 1971) and Australia (Ironsides & Matthews, 1975). It seems that workers exposed to fine wood dust, for instance furniture makers, are particularly at risk.

In order to ascertain to what extent carcinoma of the nose and the paranasal sinuses is associated with dusty occupations in Sweden, the cancer registry of the National Board of Health and Welfare was consulted as information source. This registry receives reports on newly detected cases of cancer and related diseases from the whole country. The registry material is based upon independent reports from physicians, pathologists and cytologists. Reporting to the registry is statutory and in practically all hospitals a matter of routine. Reports from clinical pathologists can be regarded as 95-100% complete.

MATERIALS AND METHODS

The analysed cases of carcinoma of the nose and the paranasal sinuses are based on registry reports from 1961-70.

The material was divided into two groups, the first consisting of cases of adenocarcinoma from 1961 until 1971, and the second of cases of squamous cell and poorly differentiated carcinoma from 1965 until 1971.

The first group consisted of 36 men and 10 women, the latter of 127 men and 85 women. All hospitals and physicians who reported to the registry were contacted and all subjects still surviving received a questionnaire.

In 1967 Acheson et al. reported a strong association of adenocarcinoma of the nose and paranasal sinuses with occupational exposure to wood dust. They found an excess of adenocarcinoma among workers in the furniture industry of High Wycombe, England where the average annual incidence rate was estimated to 0.6 per 1000. Similar findings have been reported from Belgium (Debois, 1969), Denmark (Mosbech & Acheson, 1971), Ander-

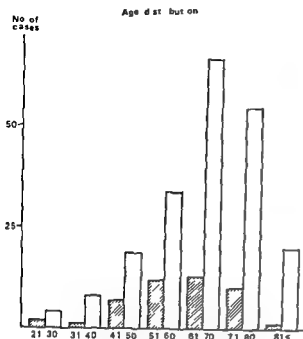


Fig 1 Age distribution. Hatched bars: adenocarcinoma; open bars: squamous cell and undifferentiated carcinoma.

was completed in more than 90% of cases. The patients stated their present and previous occupation, with special reference to various types of dusty surroundings. There were also questions concerning previous and present smoking and snuff taking habits.

The same type of questionnaire was also sent to close relatives of the deceased where these could be traced. In almost 50% of all recorded cases the questionnaires were answered. In the remainder information on previous occupations was received from the personal records of the population registry of the Swedish Church.

The total case material and age distribution is presented in Fig 1. The mean age of the adenocarcinoma subjects was 62 years and of the squamous cell and poorly differentiated cancer cases 64 years. The crude annual incidence rate is presented in Fig 2 which shows the rate of adenocarcinoma in males and the rate in males of total malignancies of the nose and paranasal sinuses.

The geographical distribution of the male adenocarcinoma cases was recorded according

to the latest known domicile. The cases were rather evenly distributed in central Sweden and there was no apparent clustering. Isolated cases only were found in the northern and most southerly parts of Sweden. Hardly any cases were found in the big cities. Most cases were localized to small towns and villages many of which had furniture industries.

RESULTS

The occupational distribution among men with adenocarcinoma is presented in Table I. Of 36 cases the questionnaire was answered in 18. As many as 19 men (53%) had evidently been joiners and at least 12 of these had been cabinet makers. Among other occupations represented 3 persons had worked in the flour industry.

The period of exposure to wood dust among the joiners is established in 9 cases and is shown in Fig 3. The arrows indicate the time of diagnosis. In a few other cases the time of exposure is more uncertain with an obvious latency period of 5 to 30 years. In all cases the duration of exposure is 25 years or more.

Most joiners in this survey had been working with different species of hardwood such as oak, teak, mahogany and birch and no one had exclusively handled softwood such as spruce or pine.

The smoking and snuff taking habits were recorded from the questionnaires only and the pattern was no different from the findings in a general survey. There were two snuff takers among the joiners.

The occupations of the males with squamous cell and poorly differentiated carcinoma are recorded in Table II which shows that questionnaires were returned in 67 cases of 127. As is seen no one occupational group dominates. However many of the occupations recorded are associated with a dusty surrounding and we found a few subjects who had worked with stone drilling, textiles, leather and flour. The number of cases is too small to be of any certain significance. The joiners in



Fig 2 Crude annual incidence rate in males

this group represented only 4% of the total number of cases

Information on the occupations of the females was scarce and it was not possible to trace the occupations of the 10 female cases of adenocarcinoma. Table III shows the distribution of occupations among the 85 females with squamous cell and low differentiated carcinoma. Only 31 cases answered the questionnaires. The occupations with exposure to organic dust such as textile work, leather and flour handling are well represented in this group. However, the bulk of the cases either come under the heading 'miscellaneous', which covers housekeeping, secretarial work, etc., or else the heading 'unknown'.

DISCUSSION

Following the reports from England and other countries, we felt a need to investigate whether there is an association of cancer of the nose and paranasal sinuses with exposure to wood dust in Sweden. At the same time we could seek other types of occupation with environmental dust possibly associated with cancer of the nose.

Table II Exposure to wood dust and to vapours in 10 woodworkers

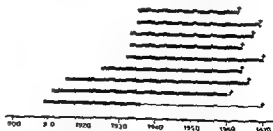


Fig 3 Time of exposure to wood dust and vapours in 10 woodworkers

Table I Occupation of males with adenocarcinoma 1961-70

Occupation	No	Percentage	Questionnaire +
Owners	14	53	10
Building workers	1	3	-
Farmers	6	17	4
Transport workers	3	8	1
Flour workers	3	8	2
Miscellaneous	4	11	1
Total	36	100	18

Table II Occupation of males with squamous cell and poorly differentiated carcinoma 1965-70

Occupation	No	Per centage	Questionnaire+
Joiners	5	4	2
Other woodworkers	8	6	5
Building workers	10	8	6
Fitters	23	18	12
Farm labourers	23	18	12
Stone drillers	3	2	3
Transport workers	6	5	6
Textile workers	6	5	2
Flour workers	1	1	-
Leather workers	1	1	-
Miscellaneous	25	20	14
Unknown	16	13	-
Total	127	101	62

The cancer registry of the National Board of Health and Welfare in Sweden is well established and is intended to cover at least 95% of all new cancer cases in Sweden. A possible way to study the problem was to analyse a case material from the registry registers. We could not, however, obtain any information from the registry regarding the domicile, occupation and survival of the patients. There is a delay of about 5 years from the time of diagnosis until processed information from all reported cancer cases is available at the cancer registry, being the reason why only cases diagnosed before 1971 were analysed.

It has been discussed whether it is in accordance with ethical practice to make direct contact with patients whose names and identification have been extracted from a computerized cancer registry. We therefore informed all physicians who had been in charge of the patients about the project and asked for permission to contact their patients. In fact two cases had to be withdrawn from the study due to a refusal from one doctor. Through the physicians, the hospitals and the population registry of the Swedish Church we obtained information about the domicile of the patients still alive, and on dates of death.

Furthermore, we discovered that the population registry had personal records where the occupation of the deceased was noted during his adult life. Unsuccessful attempts were made to trace the occupations of the patients through the Swedish Census. The questionnaires distributed were generally appreciated and we did not experience any negative attitude to them.

In accordance with previous reports and judging from our preliminary investigation it seemed relevant to separate the cases of adenocarcinoma from the cases of squamous cell and poorly differentiated carcinoma. The case material shows a male/female ratio of 3.6:1 for adenocarcinoma and 14:1 for squamous cell and poorly differentiated carcinoma. The crude annual incidence rate of males (Fig. 2) shows no secular trend, especially regarding adenocarcinoma. The geographical distribution of male cases of adenocarcinoma does not show any clustering which may be due to the fact that there are a number of small furniture firms scattered over a large part of Sweden. Another cause may be that some of the males were retired at the time of the diagnosis and had moved from the area in which they had been working.

The occupational group of males with adenocarcinoma (Table I) is dominated by joiners who made up 53% of all male cases. This number demonstrates convincingly that there is an association between adenocarcinoma in males and the joinery occupation. According

Table III Occupation of females with squamous cell and poorly differentiated carcinoma 1965-70

Occupation	No	Per centage	Questionnaire+
Textile workers	8	9	6
Leather workers	3	4	3
Farm labourers	6	7	2
Flour workers	3	4	2
Miscellaneous	48	56	18
Unknown	17	20	-
Total	85	101	31

In previous reports, it is reasonable to suggest that this association depends on the exposure to wood dust. From the questionnaires received we could not draw any conclusions regarding the type of wood involved. Most joiners had worked with different species of wood including hardwoods. One case only had worked almost exclusively with birch and pine. The question remains open, whether dust from softwoods such as pine and spruce might be associated with carcinoma.

It appears that the exposure period to the dust must as a rule cover several years, which is in accord with the findings of other investigators (Acheson et al, 1968, Andersen, 1975, Gignoux et al, 1971). The period from the last exposure to the time of the diagnosis appeared to vary from 0 to 30 years or more. There was no indication that smoking or snuff taking habits had any influence on the development of carcinoma of this type.

In order to calculate the relative risk for joiners we have tried to obtain adequate figures on the number of males actually occupied with this kind of work. The Woodworkers Union in Sweden has an estimation of 25 000 working in the wood industry, with exposure to wood dust. This number has not changed significantly during the last 40 years. At the 1960 Swedish Census, the number of males occupied in the furniture industry was 17 000. These figures give us a relative risk of 165 and 109 for joiners and furniture workers respectively as a raw estimate. In calculating this it is assumed, naturally, that the reference population consisting of males otherwise occupied is not exposed to wood dust and that other factors giving rise to a risk for this tumour are evenly distributed in the population. We also disregarded persons not traced and—in so doing—assumed the proportion of cases to be identical among the non-traced. Furthermore it is assumed that the populations compared are constant during the period of time covered by the study. Thus, a raw estimate is based on a series of assumptions some of

The calculated risk ratio is therefore given only to demonstrate the order of risk magnitude. Due to the uncertain and probably overestimated numbers the relative risk may accordingly be considerably higher than calculated.

Among the occupations represented by the males with adenocarcinoma, flour workers should be noticed, but their number is too small to be of any certain significance. The duration of exposure in these cases varied extensively.

The present investigation of the occupation of cases with squamous cell and poorly differentiated carcinoma of the nose and paranasal sinuses was carried out in order to find out whether joiners or other workers exposed to dust were at risk (Table II). Although there is no occupational group dominating the material, we found a great number of workers with occupations where exposure to organic dust is common. However, it is not possible to draw any conclusion as to a direct association with cancer. There were only 5 joiners and 8 other woodworkers among 127 male cases, which should be compared with the large number of joiners with adenocarcinoma.

It appeared difficult to obtain information on previous occupation from women with nasal carcinoma. There were 10 cases of female adenocarcinoma, one had been a textile worker, 2 had been part time farm labourers and the remaining 7 had no definite occupation recorded. Women with squamous cell and poorly differentiated carcinoma (Table III) had been working mainly in environments without known exposure to dust but there was a small number of women whose occupations had been connected with textiles, leather and flour.

The present investigation shows that woodworkers in Sweden who have been exposed to wood dust for a long period run a greater risk of developing adenocarcinoma of the nose and paranasal sinuses than other in the population, which is a contrast to the reports from England and other

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ZUSAMMENFASSUNG

Beim Studium des Zusammenhanges von Karzinom der Nase und Nebenhöhlen mit berufsmaßiger Exposition von Staub diente das Cancerregister der schwedischen Sozialverwaltung als Informationsquelle. Das Material wurde in zwei Gruppen eingeteilt. Die erste Gruppe umfaßt sämtliche Fälle von Adenokarzinom 1961 bis 1971, die zweite Gruppe sämtliche Fälle von Plattenepithel- und undifferenzierten Karzinomen 1965 bis 1971. In der ersten Gruppe waren 36 Männer und 10 Frauen, in der zweiten 127 Männer und 85 Frauen. Die Information über ausgeübte Berufe war durch Fragebogen und aus dem Personenstandsregister ersichtbar. Die vorherrschende Berufsgruppe der Männer mit Adenokarzinomen waren Tischler (19 Fälle, 53%). Wenigstens 12 von diesen waren Mobeltischler. Die Exponierungszeit für Holzstaub bei Tischlern war in 9 Fällen bekannt und in den übrigen Fällen geschätzt. Bei jedem der Patienten war die Exponierungszeit nicht weniger als 25 Jahre. Die Tischler arbeiteten mit mehreren Holzsorten. Keine Berufsgruppe der Männer mit Plattenepithel- und undifferenziertem Karzinom war dominierend, auch wenn mehreren Berufen verschiedene Sorten organischen Staubes im Arbeitsmilieu vorkamen. Bei einigen Frauen wurden Berufe mit organischem Staub, wie bei Textil-, Leder- und Getreidearbeit, vorgefunden. Die Untersuchung zeigt, daß die Mehrzahl der Männer mit Adeno-

karzinom in Nase und Nebenhöhlen im Berufsleben dem Holzstaub ausgesetzt waren.

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OCCURRENCE AND DISTRIBUTION OF VIP NERVES IN THE NASAL MUCOSA AND TRACHEOBRONCHIAL WALL

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Abstract Nerves displaying vasoactive intestinal peptide (VIP) immunoreactivity were detected in the upper respiratory tract of guinea pigs, rabbits and cats. VIP nerves were numerous in the cat, less numerous in the rabbit and rare in the guinea pig. In the nasal mucosa, fine varicose VIP nerves were found to surround nasal glands and small blood vessels. In the tracheobronchial wall, VIP nerves were observed around seromucous glands, blood vessels and smooth muscle. Ganglia located in the walls of the trachea and main bronchi contained clusters of VIP immunoreactive nerve cell bodies, conceivably representing the origin of the VIP fibres found in this region.

and distribution pattern of VIP nerves in the upper respiratory tract, with emphasis on the nasal mucosa and tracheobronchial wall.

MATERIAL AND METHODS

Adult guinea pigs, rabbits and cats were used, 5 of each species. Guinea pigs were killed by exsanguination under diethyl ether anaesthesia. Rabbits were killed by an overdose of pentobarbital sodium, and cats by exsanguination under light pentobarbital sodium anaesthesia. Specimens were taken from the nasal mucosa, epipharynx, mesopharynx, larynx and from the tracheobronchial tree. All specimens were frozen to the temperature of liquid nitrogen in a propane-propylene mixture and freeze dried. They were then fixed by exposure to diethylpyrocarbonate vapour at 55° for 3 hrs (Pearse et al, 1974), and embedded in paraffin *in vacuo*. Sections were cut at 5 µm and deparaffinized in xylene.

For the demonstration of VIP immunoreactivity the sections were subjected to an indirect immunofluorescence method (Coons et al, 1955) or to the peroxidase-antiperoxidase (PAP) procedure (cf Sternberger, 1974). VIP antiserum (code no. 5603) was kindly supplied by Drs J Fahrenkrug and O Schaffalitzky de Muckadell, Department of Clinical Chemistry, Bispebjerg Hospital, Copenhagen, Denmark. The antiserum has been described in detail elsewhere (Fahrenkrug & Schaffalitzky de Muckadell, 1977). It was

vasoactive intestinal peptide (VIP) isolated by Said & Mutt in 1970 has been generally regarded as a hormone (Grossman, 1974). However, recent immunohistochemical studies have revealed that VIP occurs in neurons. VIP nerves are numerous in the oesophagus (Uddman et al, 1978a) gastro-intestinal tract (Uddman et al, 1976, Larsson et al, 1976, Sundler et al, 1977, 1978) and genito-urinary tract (Alm et al, 1977; Larsson et al, 1978b).

Both the nasal mucosa and the tracheobronchial wall are richly supplied with adrenergic nerves (Dahlström & Fuxe, 1965; Dahlström et al, 1966; Anggård & Densert, 1974). There is also a rich supply of acetylcholinesterase (AChE) positive nerves in both these locations (Ishii, 1970; Mann, 1971). In addition, electrophysiological and pharmacological studies indicate the presence of non-cholinergic, non-adrenergic nerves in the respiratory tract (cf Burnstock, 1975).

The present report describes the occurrence

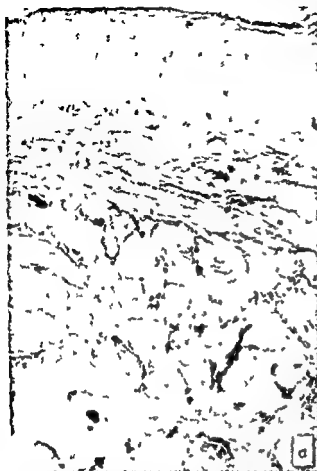


Fig. 1 Cat nasal mucosa, maxilloturbinal area. Immunohistochemical demonstration of VIP. (a) PAP procedure. Numerous VIP immunoreactive nerve terminals running close to acini of seromucous glands. Surface epithelium



at the top of figure ($\times 200$) (b) Sect on through seromucous gland. Immunofluorescent VIP nerves among acini as well as around blood vessel (arrows) ($\times 150$)

tion 1:80 for the immunofluorescence procedure, and the site of the antigen-antibody reaction was revealed by fluorescein isothiocyanate (FITC) labelled sheep anti rabbit IgG (SBL Stockholm Sweden) diluted 1:20. Immunofluorescence was observed in a Leitz Orthoplan fluorescence microscope equipped with an epi illumination system. An HBO 200 Hg lamp served as light source and filters were selected to give peak excitation at 490 nm (standard filter setting no. 3).

For the PAP procedure the VIP antiserum used was diluted 1:5600. PAP complex (Cappel Laboratories, Downingtown Pa, USA) was used in dilution 1:320. The sections were examined in a light microscope. Sections in

cubated with antiserum inactivated by the addition of excess antigen (100 μ g of pure porcine VIP per ml diluted antiserum) served as controls.

RESULTS

Nerve fibres displaying VIP like immunoreactivity were detected throughout the upper respiratory tract. The immunoreaction was blocked by adding VIP to the antiserum. Generally speaking, VIP nerves were most numerous in the cat, less numerous in the rabbit and rare in the guinea pig. The following description of the distribution of VIP nerves applies to the cat, the distribution pattern was similar in the other species examined.

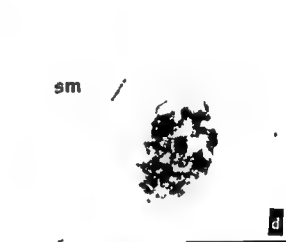
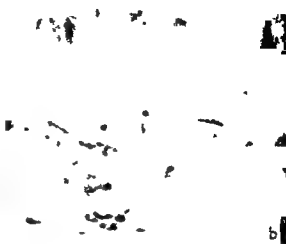


Fig. 2 Cat bronchial wall. Immunohistochemical demonstration of VIP. (a) PAP stained section. VIP immunoreactive nerves are present among acini of submucosal gland ($\times 150$). (b) VIP immunofluorescent nerves among acini of submucosal gland ($\times 300$). (c) VIP nerves in sub-

epithelial connective tissue and around bundle of smooth muscle (SM) ($\times 300$). (d) Cluster of VIP immunoreactive nerve cell bodies close to smooth muscle bundle (SM). Note single beaded VIP nerve terminal within smooth muscle ($\times 300$).

Nasal mucosa

The maxilloturbinal area received a particularly rich supply of VIP nerves; they were less numerous in the septum and upper parts of the nasal cavity. Fine varicose VIP nerves surrounded the nasal glands, forming dense plexa around the acini (Fig. 1a). In addition, VIP nerves were associated with fairly thick-walled blood vessels (Fig. 1b). Nerve trunks occurring in the connective tissue were occasionally seen to contain immunoreactive

nerve fibres intermingled with non-reactive ones.

Epipharynx, mesopharynx and larynx

In all areas examined, scattered VIP nerves were seen around seromucous glands and small blood vessels.

Tracheobronchial wall

VIP nerve fibres were most numerous in the upper parts of the

whereas they were more rare in the lower parts. Fine varicose VIP nerves formed plexa around submucosal glands and small blood vessels (Fig 2a, b). VIP nerves were also observed close to and within smooth muscle bundles in the tracheobronchial wall (Fig 2c, d). Single or clustered nerve cell bodies displaying VIP immunoreactivity were occasionally observed close to the smooth muscle (Fig 2d).

DISCUSSION

This study has revealed VIP immunoreactive nerves to be a regular constituent of the mammalian upper respiratory tract. The nerves were associated with smooth muscle, seromucous glands and blood vessels. The presence of immunoreactive nerve cell bodies in ganglia within the tracheobronchial wall suggests that at least some of the VIP nerves are intrinsic to the organ, representing short peptidergic neurons. However, it cannot be ruled out that some VIP fibres in the upper respiratory tract may originate from distant ganglia.

In the nasal mucosa and tracheobronchial wall, all adrenergic nerves (Dahlstrom & Fuxe, 1965, Dahlstrom et al, 1966) as well as AChE-positive nerves, interpreted as cholinergic (Ishii & Toriyama, 1972, Grote et al, 1975, Mann, 1971), have been observed in association with smooth muscle, seromucous glands and blood vessels. In the nasal mucosa however, the glands seem to receive a much less prominent proportion of the adrenergic nerves (Ånggård & Densert, 1974).

VIP nerves have recently been found in several types of exocrine glands, such as the salivary glands and the pancreas (Bryant et al, 1976, Larsson et al, 1976, Sundler et al, 1978, Uddman et al, 1978b). In the pancreas, VIP is known to stimulate secretion (Said & Mutt, 1972). Since VIP nerves occur around the seromucous glands of the nasal mucosa and the tracheobronchial wall, it is conceivable that they participate in regulating the secretory activity.

While vasoconstriction is thought to be

mediated by adrenergic nerves, the mechanism of vasodilatation is less well understood. Evidence for the occurrence of non adrenergic, non cholinergic inhibitory nerves in the wall of blood vessels in various locations including the nasal mucosa, have been presented (Ånggård, 1974, cf Burnstock 1975). VIP is known to be a potent vasodilator (Said & Mutt, 1970) and it is conceivable that the VIP nerves associated with blood vessels in the upper respiratory tract are involved in the regulation of local blood flow.

Electrophysiological and pharmacological studies have indicated the presence in tracheal smooth muscle of inhibitory nerves which are non adrenergic and non cholinergic (Coleman 1973, Coburn & Tomita 1973). These inhibitory nerves may be identical with VIP nerves. However, by immunohistochemistry another peptidergic nerve system storing substance P, has been described in the respiratory tract. Hokfelt et al (1975) found such nerves in the nasal mucosa of rat and Nilsson et al (1977) observed substance P nerves within tracheobronchial smooth muscle of the guinea pig. Substance P was found to cause a dose-dependent increase in the tracheobronchial smooth muscle tone (Nilsson et al 1977). In view of our finding of VIP nerves within and around the tracheobronchial smooth muscle and of the potent relaxant action of VIP on smooth muscle in other locations (Larsson et al, 1976, Uddman et al, 1978), the relaxation of adrenergically denervated tracheal muscle induced by electrical field stimulation (Coleman & Levy, 1974) may therefore reflect the action of locally released VIP rather than substance P (see also Said et al, 1974).

In conclusion, the distribution pattern of VIP nerves in the respiratory tract and the above mentioned pharmacological effects of VIP suggest that such nerves are implicated in at least three physiological actions: relaxation of tracheobronchial smooth muscle, stimulation of secretion from seromucous glands and enhancement of regional blood flow.

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ZUSAMMENFASSUNG

Nerven die VIP (vasoactive intestinal peptide) Immunoreaktivität aufweisen wurden in den Luftwegen der oberen Atmungsorgane von Meerschweinchen, Kaninchen und Katzen entdeckt. Die VIP Nerven waren zahlreich bei Katzen, weniger zahlreich bei Kaninchen und selten bei Meerschweinchen. In der Nasenschleimhaut wurden feine vaskuläre VIP Nerven gefunden die die Drüsen der Nase und kleine Blutgefäße umgeben. In den Wänden der Trachea und Bronchen wurden VIP Nerven in der Umgebung von seromukösen Drüsen, Blutgefäßen und glatter Muskulatur bemerkt. Ganglien in den Wänden der Trachea und der Hauptbronchien enthielten Ansammlungen von immunoreaktiven Nervenzellkörpern, die vermutlich den Ursprung der in diesem Bereich nachgewiesenen VIP Nerven darstellen.

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CONCENTRATION AND DISTRIBUTION OF HEAVY METALS IN NASAL MUCOSA OF NICKEL-EXPOSED WORKERS AND OF CONTROLS, STUDIED WITH ATOMIC ABSORPTION SPECTROPHOTOMETRIC ANALYSIS AND WITH TIMM'S SULPHIDE SILVER METHOD

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Abstract Biopsy specimens from nasal mucosa of 30 nickel-exposed individuals and 6 controls were analysed by atomic absorption spectrophotometry to determine the content of nickel copper cobalt zinc and iron. Timm's sulphide silver staining method was used for visualizing heavy metals in cryostat sections of biopsy material from each individual. The purpose of the investigation was to study the sulphide silver staining pattern in nasal mucosa of nickel-exposed individuals and controls and to establish whether variations in heavy metal concentrations especially nickel affect the histochemical pattern in the mucosa. No consistent differences were found in the histochemical pattern between biopsies with high and low concentrations of nickel or any of the other metals. Some difference in epithelial types between specimens from the nickel-exposed group and the control group was seen but the staining pattern was quite similar for corresponding epithelial types in the two groups. Two nasal carcinomas from nickel workers were virtually unstained with the sulphide silver staining method.

The carcinogenic effect of nickel on experimental animals has been demonstrated by several investigators (Hueper, 1952 1958, Hueper et al., 1962, Sunderman et al. 1975 Sunderman 1976).

Epidemiological studies in several nickel refineries have revealed an association between nickel exposure and development of nasal carcinomas (Morgan 1958 Mastromatteo, 1967, Doll et al. 1970 Pedersen et al. 1973, further references in Sunderman 1976). Torjussen et al. (1976) recently demonstrated the correlation between nickel exposure and develop-

ment of epithelial dysplasia, including carcinoma, of the nasal mucosa.

An increased nickel content of the nasal mucosa among nickel workers has been found in an ongoing study (Torjussen et al.). It is important to determine the distribution of this metal between the different tissue components of the mucosa. Although several histochemical techniques have been described for demonstration of nickel and other heavy metals (Pearse 1972), most of them lack specificity (see Torjussen & Haug 1978). The sulphide silver method of Timm (1958) is probably the most sensitive method, but cannot be used to distinguish between different heavy metals. Nevertheless, comparison of the sulphide silver staining pattern in specimens with normal and elevated nickel concentrations might give a clue to the site of the nickel deposition.

The purpose of the present investigation was to study the concentration of nickel, copper, cobalt, zinc and iron in the nasal mucosa of nickel-exposed and unexposed individuals and to investigate whether variations in these concentrations especially the nickel concentrations affect the sulphide silver pattern.

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Table II Mean values (\bar{M}), standard deviations ($S D$) and coefficients of variation ($CV\%$) metal concentrations ($\mu\text{g}/100 \text{ g wet wt}$) in specimens from nasal mucous membrane of n exposed individuals, grouped according to work categories, and of controls

Metals	Work categories								
	Electrolysis (E)			Roasting/smelt (R/S)			Other work (O)		
	\bar{M}	$S D$	$CV\%$	\bar{M}	$S D$	$CV\%$	\bar{M}	$S D$	$CV\%$
Ni	270	221	82	630	673	107	124	88	80
Cu	480	290	60	627	464	74	460	291	63
Zn	2 353	1 823	77	4 657	2 480	53	2 799	3 079	110
Fe	19 552	9 915	51	25 943	9 456	36	28 737	14 967	52

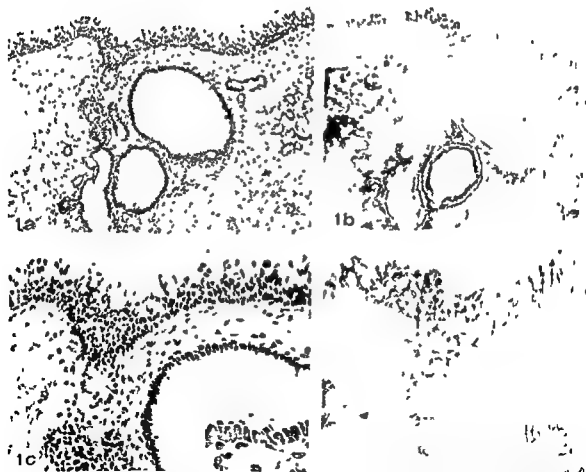


Fig 1 (a b c, d) Cryostat sections ($8 \mu\text{m}$) of specimen from nasal mucous membrane not exposed to nickel (a c) Stained with hematoxylin eosin and (b d) sulphide silver stained Stratified columnar epithelium Strong cytoplasmic staining of the basal 1-3 cell layers (b d) Arrows on strongly stained cells ($\times 100 \times 200$) (e f) Glandular duct epithelium (g) Coarse dark are clearly seen in apical cytoplasm (gr) ($\times 450$)

with the same type of epithelium as the corresponding surface (*d*, Fig 7a)

Sulphide Silver Staining Pattern

Surface epithelium

(1) *Pseudostratified and stratified columnar epithelium* A basal zone of 1-3 cell layers regularly showed a strong cytoplasmic staining (Figs 1*b*, *d*, 2*b*) In weakly stained sections the cytoplasmic stain was granular and there were often differences in stainability between individual cells (Figs 1*d*, 2*b*) In strongly stained sections the zone, except for the nuclei, appeared uniformly black (Figs 1*b*, *d*, 2*b*) The nuclei were largely unstained, containing only a few black granules (Fig 2*b*)

In general the superficial zone above the basal cell layers showed a moderate, finely granular, cytoplasmic staining (Figs 1*d*, 2*b*), while some scattered cells were distinguished by a content of larger grains (arrows Fig 1*d*) The secretory vacuoles of goblet cells were unstained (*g*, Fig 2*b*)

(2) *Stratified cuboidal epithelium* This epithelium usually showed the same staining as stratified columnar epithelium However, in areas of gradual changes between stratified

Nickel-exposed individuals			Controls		
	SD	CV%	M	SD	CV%
1	434	123	21	16	76
1	339	65	506	488	96
1	2360	77	2117	1289	61
1	10886	48	8067	8679	108

most of the specimens as shown in Table III, as made on the basis of the predominant epithelium

Stroma

In most specimens this consisted of a normal, loose connective tissue with variable infiltration of mononuclear and scattered cells (aspirates, Fig 9a)

Glands

Variable amounts of serous and mucous glands were found in most specimens Larger, superficial, glandular ducts were usually lined

Table III Epithelial classification of biopsies from nasal mucosa of 30 nickel exposed and 6 non-exposed individuals

Epithelial type in the section	Number of sections (%) from persons	
	Exposed to nickel	Not exposed to nickel
1 Pseudostratified or stratified columnar epithelium	-	-
2 Stratified cuboidal epithelium and some columnar epithelium	2 (7%)	1
3 Stratified cuboidal epithelium	13 (43%)	4
4 Stratified cuboidal epithelium and some columnar mixed or squamous epithelium	11 (37%)	1
5 Mixed stratified cuboidal/stratified squamous epithelium and some cuboidal or squamous epithelium	1 (3%)	-
6 Stratified squamous epithelium and some mixed or cuboidal epithelium	3 (10%)	-

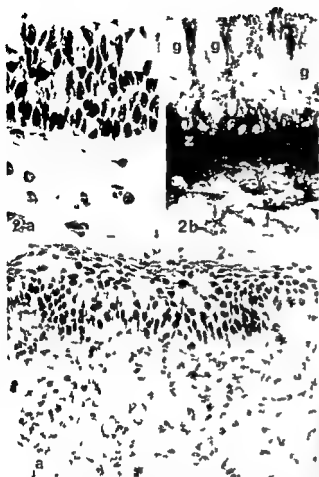


Fig 2 (a, b) Enlarged fields from the same sections as in Fig 1a, b. In (b) the superficial epithelial cells show finely granular cytoplasmic staining. Goblet cells (g) are unstained. The cytoplasm of the basal cells shows numerous confluent large black grains. Nuclei are virtually unstained but contain a few coarsely stained granules. Basal cell layers together with the subepithelial connective tissue form a broad, uniformly black band (z). Star-shaped cells (arrows) with dense staining of the cytoplasm and of slender processes are seen in the stroma ($\times 450$).

Fig 3 Sulphide silver stained cryostat section ($16 \mu\text{m}$) of specimen no. 1575 (see Table 1). Stratified cuboidal epithelium is stained like stratified columnar epithelium (see Figs 1b, d and 2b). The subepithelial connective tissue consists of a meshwork of stained fibre bundles

cuboidal and mixed cuboidal/squamous epithelium or stratified squamous epithelium, the positive sulphide silver staining often disappeared before the change in epithelial type (Fig 5a, b).

(3) *Mixed stratified cuboidal/stratified squamous epithelium*. This intermediate type of epithelium ordinarily stained weakly like



oriented parallel to the basement membrane. Polygonal cells with cytoplasmic staining among the fibres (arrows). Nuclei lightly stained ($\times 450$).

Fig 4 (a, b) Cryostat sections ($8 \mu\text{m}$) of specimen no. 1575. (a) shows a peculiar loose spongy appearance due to numerous spaces that separate the cells from each other (arrows). (b) shows the surface epithelium in (b) staining like the squamous epithelium illustrated in Fig 11b. Strong staining of single cells and aggregates of cells in the basal epithelial layers (arrowheads). Superficially, two cells are occasionally strongly stained (arrows). Fig 4b ($\times 200$).

squamous epithelium. When it showed staining of the basal cells, this was generally weaker than in the already described types of epithelium (Fig 4b).

(4) *Stratified squamous epithelium*. In this epithelium both the superficial and the deep layers remained virtually unstained (Figs 8b, 9b, 10b). However, some single cells in the

basal layers showed a weak, finely granular, cytoplasmic staining. Occasionally, superficially situated cells were similarly stained, as found in the mixed stratified cuboidal/stratified squamous epithelium.

aggregates of cells in the most basal layers of the epithelium (Fig 11b)

Stroma

Numerous polygonal, stellate or fusiform cells showed dense staining of the cytoplasm (Figs 2b, 3, 9b) and of long, slender processes, forming a meshwork between the cell bodies (Fig 3)

A finely grained intercellular staining was also seen. In part this was associated with fibre bundles (Fig 3), in part more diffusely distributed. Clefts, capillaries and other lumina often appeared to contain a stainable fluid (arrows, Fig 9b)

The subepithelial connective tissue below columnar or cuboidal epithelium often stained strongly, forming a broad dark band including also the darkly stained basal layer of the epithelium (Fig 2b). The basement membrane in its classical light microscopic sense, was nevertheless often discernible (arrow, Fig 2b). The finely grained staining associated with fibre bundles was often somewhat enhanced in the subepithelial connective tissue layer perhaps reflecting a higher density of fibres (Fig 3)

Beneath squamous epithelium the subepithelial connective tissue stained more like the remaining stroma

Mononuclear cells showed little staining (Fig 9a, b)

Glands

Glandular acini were generally unstained (as in Figs 7a, b). Stratified epithelium in larger ducts usually stained like the surface epithelium (d in Fig 7b). In simple columnar duct epithelium coarse dark grains were found in the apical cytoplasm (Fig 1f)

Carcinomas

The tumour tissue showed epithelium that was virtually devoid of sulphide silver staining infiltrating in a strongly stained connective tissue stroma (Fig 12a, b)

Comparisons of the Sulphide Silver Staining between Epidemiologically and Chemically Defined Groups of Specimens

Total staining The ratings of total staining in one set of sections correlated well both for each observer and between observers ($r=0.7$ and 0.9 , respectively, $P<0.01$). Moreover, similar comparisons between adjacent ($8\text{ }\mu\text{m}$) sections from each specimen, stained in different batches, showed good correlation ($r=0.7$). However, there was no correlation between the ratings of one set of $8\text{ }\mu\text{m}$ and one set of $16\text{ }\mu\text{m}$ thick sections ($r=0.27$). These two sets, besides being of different section thickness, were from levels spaced about $250\text{ }\mu\text{m}$ apart. It is possible that the lack of correlation here reflects the heterogeneity in the blocks. A comparison between specimen groups made on the basis of 'total staining' and chemical or epidemiological data, therefore seemed meaningless.

Distribution pattern The staining densities of the superficial and basal layers of the different types of epithelium, of subepithelial and deeper levels of the connective tissue, of the glands and of infiltrates of mononuclear leucocytes, were rated as for 'total staining' in two $8\text{ }\mu\text{m}$ sections from each specimen. We were not able to find any clearcut association between chemical and epidemiological data and the distribution and densities of the sulphide silver staining. A comparison between 11 specimens with the highest and 9 with the lowest nickel concentrations showed no difference in sulphide silver pattern, nor did similar groups based on the concentrations of the other metals on the sum of the nickel, copper concentrations, or on the iron c

The specimens of the controls did not differ in distribution pattern or total staining from specimens of the nickel-exposed group, when comparing corresponding epithelial types. The conclusion to be drawn here is that we failed to distinguish nickel from the other stainable metals with the present applied histochemical method.

DISCUSSION

Tissue concentrations of nickel, copper, cobalt, zinc and iron in nasal mucosa

The concentrations of these metals in the nasal mucosa have not been previously investigated. As regards nickel, the present data should be regarded as preliminary to a report on nickel in the nasal mucosa, plasma, and urine of nickel workers (Torjussen et al., in preparation). At this point it should be mentioned that the air in both the electrolytic and the roasting/smelting departments contains large amounts of nickel, and lesser amounts of copper. In the roasting/smelting department, nickel occurs as water-insoluble compounds (nickel sulphide and oxide), while compounds with high solubility predominate in the electrolytic department (nickel chloride and sulphate). This difference in type of nickel exposure probably contributes to the significant differences in mean nickel concentration in nasal mucosa between the roasting/smelting department and the electrolytic department.

The high coefficient of variation for nickel in the nasal mucosa probably reflects the variable degree of exposure to this metal in the material. However, the coefficients of variations for copper, iron and zinc are also fairly high. Moreover, the biopsies differ considerably with respect to the volume fractions of the different tissue types, where the sulphide silver pattern has demonstrated that the metals have an uneven allocation. Thus, sampling variations may contribute significantly to the large variability of the metal concentrations.

Structure of nasal mucosa

Oppikofer (1906) studied the mucosa of the inferior and middle turbinate in a general autopsy material and found the same variation in epithelial types that we have described, although using other designations. The amount of glands and the degree of mononuclear leukocyte infiltration in the connective tissue varies both between and within the specimens consistent with the descriptions of Oppikofer (1906).

The present biopsies were taken from the location which deviated most from 'typical' respiratory epithelium in Oppikofer's material.

Fig 5 (a, b) Cryostat sections (8 µm) of specimen no 1555 (Table I) (a) Stained with hematoxylin-eosin and (b) with sulphide silver method. Fold of mucosa lined with thick stratified cuboidal epithelium (x). Transition to stratified squamous epithelium near the top of the fold (y). The basal epithelial cell layers in the bottom of the fold show strong sulphide silver staining, which disappears somewhat before the epithelium changes its appearance into squamous epithelium (area between arrows) (x150).

Fig 6 (a, b) Cryostat sections (16 µm) of specimen no 1573 (Table I) (a) Stained with hematoxylin-eosin and (b) with sulphide silver method. Obliquely sectioned thickened stratified cuboidal epithelium penetrated by connective tissue papillae (p) (x150).

Fig 7 (a, b) Cryostat sections (8 µm) of specimen no 1539 (Table I) (a) Stained with hematoxylin-eosin and (b) with sulphide silver method. Surface epithelium (s) and underlying connective tissue (c) (x150).

Fig 8 (a, b) Cryostat sections (8 µm) of specimen no 1552 (Table I) (a) Stained with hematoxylin-eosin and (b) with sulphide silver method. Counterstained with toluidine blue. Stratified squamous epithelium. Surface epithelial cells are not sulphide silver stained (only counterstained). Neither is the immediately subjacent subepithelial connective tissue (x150).

Fig 9 (a, b) Cryostat sections (8 µm) of specimen no 1552 (Table I) (a) Stained with hematoxylin-eosin and (b) with sulphide silver method. Counterstained with toluidine blue. Stratified squamous epithelium. Surface epithelial cells are not sulphide silver stained (only counterstained). Neither is the immediately subjacent subepithelial connective tissue (x150).

(b) is due solely to the counterstaining. The epithelial cells are demarcated by distinct light cell borders in both sections. Stellate cells (arrowheads) with dense cytoplasm and light nuclei are seen in the stroma. Capillaries and other lumina often appear to contain a stainable fluid (arrows). Lymphoid infiltrates (asterisks) in the stroma show virtually no sulphide silver staining (x150).

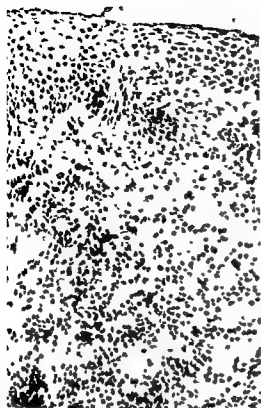






Fig 10 (a b) Cryostat sections (8 μ m) of specimen no 1536 (Table 1) (a) Stained with hematoxylin-eosin and (b) with sulphide silver method. Counterstained with thionin. Stratified squamous epithelium of unequal thickness penetrated by connective tissue papillae (p). The super-

ficial and deep cell layers of the epithelium are seen in (b) stained epithelial layers are seen in (b) stained sections (1536).



chemically and epidemiologically defined groups of specimens, as there are no practical procedures for quantitating and comparing staining reactions in different types of epithelium. The conclusions which may be drawn at this point, are therefore general.

The pseudostratified/stratified columnar epithelium generally showed intense staining of the basal cells and moderate to weak staining at more superficial levels. Although the staining of stratified cuboidal epithelium usually conformed to that of the columnar epithelium, there were deviations from this pattern, where the epithelium was weakly stained throughout its thickness. As such variations occurred even within one and the same section, and over very small distances along the epithelium, while the staining of the adjacent structures was uniform, they are unlikely to be artefacts of staining. All these deviations in staining pattern were from specimens of the nickel exposed group.

The stratified squamous epithelium was ely unstained. It is not known whether this of Timm staining is a general characteristic of this epithelial type, or specifically reflects the chronic irritation of the nasal mucosa. In the present study the squamous epithelium was found only in the nickel-exposed group.

The cancerous epithelium from two nasal tumours, at least one of them diagnosed as squamous cell carcinoma, was also virtually devoid of Timm staining. This seems the more interesting as speculations have been advanced that a lack of zinc may characterize certain types of cancer cells (Schrodt et al, 1964; Gyorkey et al, 1973). Voigt (1959) described a typical hormone-producing insulinoma, which did not stain with the sulphide silver staining method, even though normal Langerhans' islands contain easily stained zinc (Voigt, 1959).

As the controls did not differ significantly from the nickel-exposed group in the staining of corresponding epithelia, we conclude that the staining pattern of the columnar epithelium

and its underlying stroma is a normal characteristic of the nasal mucosa. This normal staining pattern is not drastically disturbed in the stratified cuboidal epithelium. However, the formation of more squamous like stratified epithelium (mixed stratified cuboidal/stratified squamous and clear stratified squamous epithelium), appears to entail loss of Timm stainability.

Despite its failure to visualize the abnormal nickel deposits, the sulphide silver staining pattern should form a useful basis for further investigation of the distribution of heavy metals in nasal mucosa.

ZUSAMMENFASSUNG

Biopsien der Nasenschleimhaut von 30 nickel-exponierter Individuen und 6 Kontrollen wurden mit Atomabsorptionsspektrophotometrie auf ihren Gehalt an Nickel, Kupfer, Kobalt, Zink und Eisen analysiert. Timms Silber-sulfidfarbemethode wurde benutzt um die Schwermetalle in Kryostatenschnitten sichtbar zu machen. Es war das Ziel der Untersuchung das Silbersulfidfarbemuster in der Nasenschleimhaut von nickel-exponierten und Kontrollen zu studieren und zu sehen ob Unterschiede im Schwermetallgehalt - insbesondere von Nickel - das histochemische Farbemuster beeinflussten. Es konnten keine konstanten Unterschiede im histochemischen

funden. Aber die histochemischen Farbemuster waren sehr ähnlich für die entsprechenden Epitheltypen der beiden Gruppen. Zwei Nasenkarzinome von nickel-exponierten waren so gut wie ungefärbt nach Anwendung der Silbersulfidfarbemethode.

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THE CENTRAL RHYTHM OF THE NASAL CYCLE

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Abstract The resistance to air flow of each nasal passage was recorded in 2 subjects over a period of 7 days. The cyclical changes in nasal resistance observed were very regular, with a consistent pattern apparent over the period of study. The regular changes in nasal resistance recorded under laboratory conditions may be directly related to changes in activity of an autonomic centre in the brain.

The spontaneous changes in the resistance to air flow of each nasal passage associated with the nasal cycle are believed to be regulated from a nasal centre in the hypothalamus or medulla. A hypothalamic centre regulating the cycle was first proposed by Stoksted in 1954 and in 1959 Malcolmson demonstrated stimulation of the cat hypothalamus caused a nasal vasoconstriction. A nasal cycle associated with the respiratory centre in the medulla has also been suggested as changes in nasal resistance are often associated with changes in respiration (Dallimore & Eccles, 1977). The nasal resistance is influenced by many factors in the environment but under controlled laboratory conditions one might expect the changes in nasal resistance to be directly related to an endogenous rhythm in the nasal centre. The present study is concerned with demonstrating the marked regularity of the nasal cycle under laboratory conditions and provides further evidence for a nasal centre as the regulator of the spontaneous changes in nasal resistance.

METHODS

Experiments were performed on 2 male subjects. R. E. (the author, age 28) was of British origin and had been exposed to the Indian cli-

mate in Delhi for 2 weeks prior to the experiments, and A. K. (age 25) was an Indian resident of Delhi. Both subjects were healthy and were not prone to any allergic disorders or chronic nasal or sinus infections. There were no external signs of a deviated nasal septum and no previous history of trauma to the nose or paranasal sinuses in either subject.

The resistance to air flow of each nasal passage was determined by measuring the pressure and flow characteristics associated with normal breathing and plotting the pressure against flow by display on a storage oscilloscope. The slope of the plot on the oscilloscope screen between flows of ± 10 l/min was determined by fitting a straight line to the graph and the nasal resistance was expressed in units of $\text{cm H}_2\text{O/l/sec}$ (R). Only the flow rates between ± 10 l/min were used for the calculations as at higher flow rates the air flow in the nasal passages is not laminar and the resistance is not a direct linear function of pressure and flow. In the present experiment the formula $R = \Delta P / V$ described the relationship between pressure, flow and resistance.

To measure pressure and flow a soft plastic cannula was inserted approximately 0.5 cm into each nostril. The cannulae were 22 cm in length with an internal diameter of 0.9 cm and a wall thickness of 2.5 mm.

A cannula from one nostril was connected to a pneumotachograph cone to measure air flow (V), with a differential pressure transducer (Hewlett Packard model 270) to determine the pressure change across the cone. The signal from the transducer was fed into an amplifier (Hewlett Packard 8805B) and then

played on the horizontal axis of a storage oscilloscope (Tektronix type 5103N)

The cannula from the other nostril was connected to one arm of a differential pressure inducer (Statham PM 283 TC) and this measured the nostril pressure which under the conditions of the experiment was equal to the anterior nares pressure as no air flow occurred in this cannula. The other arm of the inducer was connected to a large bore range needle inserted into the other cannula to record air flow, and the pressure was measured close to the nostril. With this arrangement the pressure change from the posterior nares to the nostril could be measured. The signal from the transducer was fed to an amplifier (Statham bridge amplifier SC 100) and then to the vertical axis of the storage oscilloscope.

With the cannulae inserted into the nostrils the pressure and flow relationships of one nasal passage during normal respiration were recorded on the oscilloscope, and then the cannulae swapped around and the other nasal passage monitored.

The resistance of both nasal passages in subject R was determined at approximately 10 min intervals throughout the day (09.00–23.00). The subjects remained in the laboratory for the duration of the experiment each day and avoided any exertion. The temperature range for the duration of the study was between 25–33°C and the relative humidity between 64–98% saturated.

RESULTS

Changes in nasal resistance of the subject R for 3 consecutive days of recording are shown in Figs 1 and 2. Both subjects had regular cyclic changes in nasal resistance with a reciprocal relationship between the resistance of left and right nostrils.

The cyclic changes in nasal resistance for subject R are shown in Fig. 1 and are remarkably similar for the 3 days illustrated. At the start of recording in the morning the left nasal passage

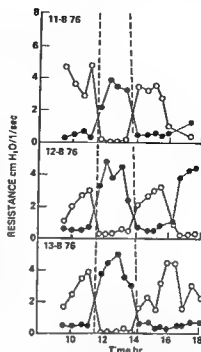


Fig. 1 Changes in nasal resistance in subject R for 3 consecutive days. \circ Left nasal passage, \bullet right nasal passage.

has a high resistance and the right nasal passage a low resistance with most of the air flow occurring through the right nostril. The changeover from right to left nostril dominance of air flow occurs at about 11.30 hr with further crossover points at 14.00 and 16.00 hr. The crossover points for 3 days of recording are joined with a straight line through the graphs to demonstrate the regularity of the cycle.

The Indian subject A.K. showed similar regular changes in nasal resistance yet the crossover points between left and right dominance showed some phase shift each day as demonstrated by the straight lines drawn through the crossover points on the three consecutive graphs (Fig. 2). In both subjects the crossover points could not be correlated with the taking of food or drink or any change in the laboratory environment.

The changes in resistance were similar for both subjects with the maximum resistance of a nasal passage varying between 4–6 cm H₂O/l/sec and the minimum resistance

0.02–1.0 cm H₂O/l/sec, depending on the phase of the cycle

The period of the cyclic changes in resistance in the two subjects varied between 1–2½ hr between crossover points depending on which nostril was dominant. Subject R E had a very regular cycle with the time periods of left or right dominance of approximately equal duration giving a crossover approximately every 2 hr.

The times of the crossover points of the graphs of the nasal cycle are tabulated in Fig 3 for the seven days of recording. Subject R E has a very regular pattern of crossover points with changes in resistance at 11.00 and 14.00 hr which are consistent for the 7 days of recording and unaffected by the weekend break on the 14th and 15th of the month. The changes in resistance which occur after 14.00 hr do not have the same regular pattern.

The crossover times for subject A K as shown in Fig 3 appear to have a random distribution on first inspection yet a regular phase in the period of the cycle is apparent on examination, similar to that observed in

the total nasal resistance calculated for both nasal passages in parallel varied according to the phase of the cyclic changes in resistance. The changes in total nasal resistance for subject R E are shown in Fig 4, where it is apparent that there is some regularity in the changes in resistance from day to day, with a range in nasal resistance from 0.06–0.64 cm H₂O/l/sec. Subject A K showed similar changes in total nasal resistance with a range from 0.21–1.14 cm H₂O/l/sec during the three days of recording.

DISCUSSION

The results demonstrate that there are regular reciprocal changes in nasal resistance in both the subjects studied. Similar results have been previously reported, but other workers have not recorded the changes in nasal resistance in the same subjects on consecutive days.

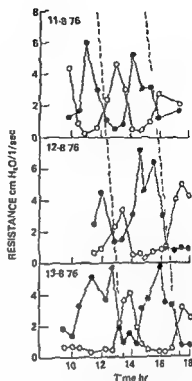


Fig 2 Changes in nasal resistance in subject A K for 3 consecutive days. ○ Left nasal passage ● right nasal passage

In subject R E the changes in resistance of each nasal passage occur at approximately the same time each day (11.00 and 14.00 hr) with no apparent phase shift. The changes in nasal resistance which occur after 14.00 hr are not as consistent from day to day as the crossover times observed at around 11.00 and 14.00 hr. If the nasal cycle was in some way connected with the time of rising from sleep and this time was consistent each day then it is possible that the cycle could be set by this time and gradually drift from the set point towards evening. The daily changes in nasal resistance which occurred at around 11.00 and 14.00 hr were not related to any intake of food or drink as meal and snack times were alternated in both subjects.

The nasal cycle of subject A K shows a significant change in the time of crossover each day yet this is probably a regular phase shift, although it would be necessary to record over a longer period to confirm this result.

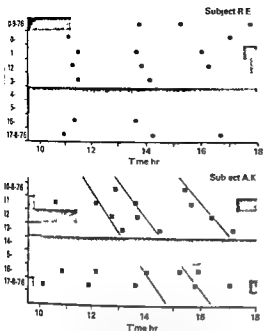


Fig 3 A plot of the crossover times of changes in nasal resistance. The shaded areas represent the periods during which recordings of nasal resistance were not made.

The phase shift each day may be explained by the relationship between the time period of the cycle and whether or not it is divisible to a 24 hr period. If the period of the cycle is exactly divisible into 24 hr then the crossover times will occur at exactly the same time each day, and if the period is not divisible into 24 hr then there will be some phase shift each day. The regularity of the nasal cycle from day to day indicates that the cyclic changes in nasal resistance occur throughout the 24 hr period and that changes in nasal resistance may continue during sleep.

The total resistance of the nasal passages to air flow has been previously stated to remain relatively constant during the reciprocal changes in the resistance of each nasal passage (Stoksted 1953). The results of the present study show that the total nasal resistance may vary according to the phase of the cycle. In subject R E the total nasal resistance ranged from 0.06–0.64 cm H₂O/l/sec and in subject A K from 0.21–1.14 cm H₂O/l/sec. This variation in total nasal resistance

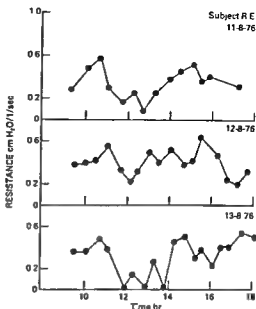


Fig 4 Changes in total nasal resistance in subject R E for 3 consecutive days.

ably associated with an undetected asymmetry of the nasal passages.

The functional significance of the cyclic changes in resistance is obscure, the changes in the state of the erectile tissue have been noted to coincide with changes in the secretory activity of the nasal mucosa (Lillie, 1923), and it is possible that there is periodic activity of the nasal mucosa in conditioning the inspired air, with one nasal passage undergoing a rest period whilst the air flow is directed through the other passage.

The vasomotor and secretory activity of the nasal mucosa is regulated by the autonomic nervous system (Ånggård & Edwall 1974, Eccles & Wilson 1974, Malm 1973), and the changes in activity of autonomic outflow which occur during the nasal cycle must be regulated centrally. The hypothalamus has been proposed as an area of the brain most likely to be involved in regulation of the nasal cycle (Stoksted 1953) and experiments on the cat have demonstrated that electrical stimulation of the hypothalamus causes a change in nasal resistance (Malcomson 1959).

the cyclic changes in nasal resistance are very regular when recorded under laboratory conditions, probably because the control of nasal resistance is then dominated by the endogenous nasal centre. Normally the activity of the nasal centre might be expected to be influenced by many factors such as, exercise and arterial $p\text{CO}_2$ (Dalimore & Eccles, 1977), emotion (Wolf, 1954) and skin temperature changes (Drettner, 1961). When changes in these factors are limited then the central rhythm of the nasal cycle is apparent and this is very regular from day to day.

Persons trained in *pranayama* (breathing exercises in Yoga) are able to alter their nasal air flow at will from one nasal passage to the other and presumably they have developed conscious control of the autonomic nasal centre. Ancient documents relating to the practice of Yoga describe changes in nasal air flow related to changes in the state of meditation and mood (Sing & Chhina, 1974) and if the nasal centre is indeed located in the hypothalamus as proposed by Stoksted then there may be a physiological basis to these ancient beliefs, with changes in mood affecting the activity of a hypothalamic nasal centre.

SUMMARY

- 1 Cyclic changes in nasal resistance have been observed in 2 subjects over a period of 7 days
- 2 The period of the nasal cycle was regular, with a consistent pattern apparent for the changes in resistance throughout the 7-day study
- 3 The total nasal resistance of each subject varied according to the phase of the nasal cycle
- 4 The regular changes in nasal resistance recorded under laboratory conditions may be directly related to changes in activity of an autonomic nasal centre in the brain

ZUSAMMENFASSUNG

Bei zwei Versuchspersonen wurde während einer Zeit von sieben Tagen der Luftstromwiderstand in den beiden Nasengängen registriert. Die beobachteten zyklischen Veränderungen des Nasenwiderstandes wiesen ein sehr regelmäßiges und während der Beobachtungszeit gleich bleibendes Muster auf. Die unter Laborbedingungen registrierten regelmäßigen Veränderungen des Nasenwiderstandes könnten in direkter Verbindung mit Veränderungen der Aktivität eines autonomen Zentrums im Gehirn stehen.

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CHARACTERIZATION OF CLINICAL TONSIL STAGES BY THEIR T-CELL COUNT

Immunopathological Description of Tonsils after a Peritonsillar Abscess

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Summary The T-cell counts in five different clinical tonsil stages are compared. No particularities were observed with the exception of the condition after a peritonsillar abscess. The differences existing between the individual stages are discussed as age-dependent. A considerable increase in T cells is found in the abscess tonsil compared to other tonsils and the T-cell count in blood. Its pathomechanism is discussed.

An important advance in immunologic research is signified by the possibility of differentiating between B lymphocytes and T lymphocytes due to different surface markers. It is now possible to analyse the immunologic stage of lymphoid cell populations and their changes. The E-rosette formation with sheep erythrocytes was used for the determination of T cells (Brain et al., 1970, Jondal et al., 1972, Lay et al., 1971).

Numerous publications are already available on the T-cell content of the tonsil (Yata et al., 1973, Bellanti et al., 1974, Brown & Greaves, 1974, Greaves et al., 1974, Greaves & Brown, 1974, Tabata et al., 1974, Watanabe et al., 1974, Yu et al., 1974, Hurtado et al., 1974, Holowiecki et al., 1975, Ishikawa et al., 1975, Morag et al., 1975, Strelkauskas et al., 1975, Alexopoulos et al., Sugiyama et al., 1976, Ellison et al., 1976). Due to different cell isolation methods, test modifications, evaluation methods and groups of patients, values are found between 21% and 74% which are actually impossible to compare one with another. Clinical aspects concerning the stage of the tonsil, however, are not considered in the

investigations. The present study aims to correlate the T-cell changes of the tonsil with several clinical conditions of the organ. For this reason we have determined the T cell count in tonsillar cell suspensions and peripheral lymphocytes in a group of 86 patients.

MATERIAL AND METHODS

Obtaining of material, cell isolation, and fine purification of lymphoid cells from tonsils and blood have already been described in detail (Siegel, 1978b) and are, for this reason, only briefly outlined here.

The tonsils were cut into pieces with scissors, and the tissue pap shaken in Hanks' solution. Tissue residues were removed by filtration through gauze, the suspension centrifuged at 160 g for 10 minutes and placed in Parker's solution.

Lymphoid cells were isolated from the suspension and blood through a gradient of Visotrac/Ficoll having the density of 1.085 in accordance with the method of Boyum (1968). Blood was taken directly before or after tonsillectomy by venipuncture and 50 IU/ml preservative free heparin was added.

E rosette assay

In accordance with the method of Jondal et al. (1972) a 50 µl suspension of 3×10^6 /ml of purified tonsillar cells or blood is

Table I *Average of the T-cell number in different disease groups*

Group	Tonsil (%)	Blood (%)	n
1	36.1±10.6	57.0±7.8	31
2	42.8±13.1	63.4±9.9	7
3	48.5±10.0	61.6±8.3	18
4	47.8±13.2	58.7±8.1	17
5	60.0±15.8	58.0±11.9	13

With the exception of Gr. 5 the differences observed in the tonsil are conditioned by age. The T cell count is always below that of blood. In group No. 5 the value is significantly above the values of the other groups and also of blood. There are no differences in T cell numbers of blood between the different groups.

cytes in Parker's solution was mixed with a 50 µl suspension of sheep erythrocytes having a cell count of 10⁸/ml in physiological saline. The sheep erythrocytes were 1-4 days old and washed twice in physiological saline directly before use. After a 5 minute preincubation at 37°C the cells were sedimented at 50 g for 10 minutes and incubated in a refrigerator for one hour. Then the cells were resuspended by careful rotation and transferred into a counting

chamber. The number of rosettes was determined by counting of 100 lymphoid cells.

RESULTS AND DISCUSSION

The group of 86 patients covered the age range 2 to 57 years. This group was divided according to the clinical stages of the tonsils as follows:

- 1 hyperplastic tonsils of children and young people (n=31) aged 2 to 17,
- 2 atrophic tonsils (n=7) of children aged 4 to 9,
- 3 chronically inflamed tonsils of adults (n=18) aged 11 to 34,
- 4 tonsils of adults with suspected focal infection (n=17) aged 10 to 40,
- 5 tonsils after a peritonsillar abscess in children and adults (n=13) aged 4 to 57 (interval tonsillectomy, for individual data see Table II).

The results of the investigations are shown in Table I in the form of averages of the individual groups. It can be seen that the tonsil T-cell count is always below that of blood with the exception for abscess tonsils. Where as the values of all groups are practically constant in blood, there are differences between

Table II *T-cells after a peritonsillar abscess*

No	Age (years)	Sex	Abscess (%)	Opposite side (%)	Peripheral lymphocytes (%)	Time between abscess and operation (weeks)
1	4	♂	49	35	50	6
2	10	♀	52	31	45	8
3	14	♂	36	19	59	6
4	15	♀	66	50	62	7
5	19	♀	88	64	58	6
6	19	♀	64	43	60	8
7	20	♂	44	35	41	9
8	22	♀	73	51	59	8
9	27	♂	43/44	—	37	5
10	30	♀	86	74	72	6
11	35	♀	75	70	73	6
12	43	♀	57	53	71	8
13	57	♀	68	64	72	6

T-cell number in the abscess tonsil and in peripheral lymphocytes. Increased values are found on the side affected by the abscess compared with peripheral lymphocytes. The contralateral side shows only partly pathological changes and the values do not exceed those of blood. The tonsillectomy was done normally at an interval of 6 weeks after the abscess.

individual clinical groups. However, the differences are not significant in groups of approximately the same age and the existing differences of groups 1 and 2 compared with groups 3 and 4 are, above all, conditioned by age and not by the clinical stages. Elsewhere we were able to demonstrate a relationship between the tonsils' T cell count and age (Siegel 1978a). The group of tonsils after a peritonsillar abscess shows a different behaviour. The group average here exceeds that of blood. The values of this group are listed individually in Table II. A distinction is made between the abscess tonsil and the non affected tonsil according to case history details. It can be seen at the contralateral tonsil frequently shows an increased T cell count, but it does not raise the values of the abscess side. The normal behaviour of Pat No 3 values in pat no 3 is presumably based on insufficient case history details and the atypical behaviour of Nos 12 and 13 can possibly explained as advanced tonsil involution in the early stages of the abscess.

An interpretation of the T cell accumulation in the abscess tonsil is possible in connection with other analytical data. Whereas exclusion of T cells is normally observed in the tonsil compared with the values of blood, the tonsil shows a contrary behaviour after an abscess. In previous investigations we were able to demonstrate a considerably reduced uridine incorporation in this stage, which was interpreted as a decrease of cell proliferation (Siegel 1978c).

A decrease in the RNA and DNA concentrations along with an increase in the protein/DNA ratio was also shown (Siegel & Ilke 1975). Since mainly B cells proliferate in the tonsil, according to the investigations of Holowiecki et al (1975) a relative concentration of T cells takes place in the abscess tonsil. We observed a reduction of the proliferative activity in the organ. An atypical composition of lymphoid cells occurs. Owing to the reduction in proliferation activity the tonsil cannot fulfil its amplifier function in the sequence

from antigen contact to immune response. This should be considered in the indication for a tonsillectomy after a peritonsillar abscess, even in children. In the groups listed in Table I, no further pathological particularities were apparent, except in the stage after a peritonsillar abscess. From this we conclude that the scattering of T cells is larger in the tonsil due to processes of natural involution than by pathological processes. The individual variation, however, is considerable.

ZUSAMMENFASSUNG

Die T Zell Zahl bei 5 verschiedenen klinischen Tonsilliten wird verglichen. Mit Ausnahme des Zustandes nach Peritonsillarabszess wurden keine Besonderheiten beobachtet und die zwischen einzelnen Gruppen bestehenden Differenzen als altersbedingt diskutiert. Bei der Abszess-Tonsille tritt eine starke Zunahme der T Zellen im Vergleich mit den übrigen Tonsillen und der T Zell Zahl des Blutes auf. Sein Pathomechanismus wird diskutiert.

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THE DNA CONTENT AND NUCLEAR SIZE IN NORMAL, DYSPLASIA AND CARCINOMATOUS LARYNGEAL EPITHELIUM

A Spectrophotometric Study

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Abstract The aim of the present study was to obtain a more objective evaluation of nuclear hyperchromasia and polymorphism in laryngeal epithelium. The method was based on a photometric assay of nuclear size and DNA content in Feulgen stained slides. The DNA content of hyperchromatic nuclei should exceed the mean for nuclei in normal epithelium by more than twice the standard deviation. In comparison with lymphocytes (2N) the DNA content in hyperchromatic nuclei corresponds to 3N. The mean DNA contents of normal dysplastic and carcinomatous laryngeal nuclei were 69, 71 and 118 U. The mean nuclear area for normal, dysplastic and carcinomatous epithelial cells were 48, 41 and 73 μm^2 . The higher the degree of atypia displayed by the cells, the greater was the variability of the DNA content and the nuclear size. The investigation shows that the method may provide a more objective basis for evaluating hyperchromasia and polymorphism.

The histopathological grading of atypia in laryngeal epithelium is based on morphological criteria that are occasionally subtle and thus vulnerable to individual differences in evaluation. A more accurate assessment of atypias is required for both diagnostic and therapeutic purposes. Nuclear hyperchromasia and polymorphism are two important parameters in the grading of atypia in squamous epithelium.

The importance of nuclear polymorphism in the malignancy grading and prognosis of laryngeal carcinoma has been well documented by Jakobsson (1973).

Nuclear hyperchromasia—that is, an enhanced stainability of the nuclei—is due to the presence of increased amounts of acidophilic substances, including DNA, RNA, histones and other proteins.

Normal nuclei may also be hyperchromatic when passing the S and G2 cell cycle phases. However, the extent to which the individual increase in DNA, RNA, histones and other proteins is responsible for the hyperchromasia observed in cells stained by the haematoxylin and eosin method has not hitherto been established. Eneroth et al (1974) also showed significantly increased DNA values in Feulgen-stained cells from an acinic cell tumour without malignant histological features.

Cells with abnormal chromosome numbers are referred to as aneuploid (or heteroploid). It would perhaps be more appropriate to refer to the chromosome constitution as hyperdiploid, hypodiploid etc, thus indicating more precisely the deviation from the diploid constitution. The chromosomes may, however, be present in multiples of the haploid number, thus giving rise to triploidy (3N), tetraploidy (4N) or higher degrees of ploidy (Köller, 1972).

The nucleoproteins consist of proteins and nucleic acids, to which they owe their staining properties. The best available histological staining method for DNA, is based on the Feulgen reaction, which is specific and does not produce staining of RNA (Feulgen & Rensenbeck, 1924; Disbrey & Rack, 1970).

The histopathological concept of polymorphism is based on nuclear size and shape of the nuclei and on the pattern of chromatin within the

ject of the study reported here was to find whether a photometric examination with determination of the DNA content and size of the nuclei in atypical laryngeal epithelia might afford a more precise foundation for grading of hyperchromasia and nuclear polymorphism

MATERIAL AND METHODS

Microscopic preparations

Nine laryngeal biopsy specimens, one from each of 9 patients, were selected and classified in the following way

Cases 1, 2 and 3 Normal squamous epithelium, with neither hyperplasia nor atypia

Cases 4, 5 and 6 Squamous dysplasia—mild, moderate and severe, respectively

Cases 7, 8 and 9 Squamous cell carcinoma moderately well differentiated

The specimens were fixed in 10% neutral buffered formalin and embedded in Paraplast Plus (Sherwood Industries)

In addition, lymphocytes were studied in smears from blood and from imprints using the Feulgen fixative

Histological sections

Sections 8 and 14 μm thick were cut with a motorized microtome with a vertical knife (Leitz microtome Model 1212)

Feulgen reaction

All specimens were stained simultaneously according to the Feulgen method (Feulgen & Rossenbeck, 1924, Disbrey & Rack, 1970) with hydrolysis in 1 N hydrochloric acid at 60°C for 12 minutes and stained with pararosaniline (C.I. 42500). The sections were mounted with Diatex (refractive index 1.485) and stored in the dark at room temperature

Photometry

The photometric measurements were carried out at the wavelength of 547 nm using a Leitz microspectrophotometer (Leitz Sinco 8 pro-

gramme), MPV II connected to a computer PDP 8E (Digital Equipment). With this equipment a rapid scanning can be performed with measuring spot of 0.25 μm^2 in area (Norstrom et al., 1975)

The accuracy was increased by making 10 registrations of transmission at each measuring spot. The absorbance was obtained by applying Beer Lambert's law using the logarithm of the mean. The area of the cell nuclei was estimated from the number of measuring spots registered within the nucleus

Conditions and errors in photometric measurements

The theoretical and practical bases of photometry have been discussed in detail by several authors (Caspersson et al., 1957, Glick et al., 1951, Leuchtenberger, 1958)

1. In photometric measurements it is essential that the absorbance be proportional to the concentration and the thickness of the absorbing substance so that Beer Lambert's law is valid

2. For the quantitation of DNA by the staining reaction in question the reaction must be a stoichiometric one (Glick et al., 1951)

3. The (distributional) error incurred by heterogeneous distribution of the absorbing substance has been calculated and critically examined by a number of workers (Glick et al., 1951, Garcia, 1962a, Leuchtenberger, 1958). For normal nuclei it has been estimated at about 1% and for carcinomatous nuclei at about 5–10% (Sandritter et al., 1960). The distributional error increases with the absorbance (Garcia, 1962b) but this error can be diminished by using a wavelength that gives a lower absorptivity (Leuchtenberger, 1958)

4. Light scatter, another source of error, was reduced by inserting a special diaphragm in the optical system

5. A high level of reproducibility of a photometric measurement is essential if measurements of the same nucleus gave a coefficient of variation of 1.02%

6. The distribution of the absorbing p

Table 1 Mean DNA absorbance (content) and area of cell nuclei and the standard deviation and coefficient of variation for 8 and 14 μm sections

	8 μm		Coefficient of variation	14 μm	
Case	Mean \pm S D			Mean \pm S D	
Absorbance(A U)					
Normal					
1	70.6 \pm 7.5	69.2 \pm 8.3	12.0%	70.9 \pm 8.9	70.3 \pm 8.6
2	68.0 \pm 8.4			69.1 \pm 7.7	
3	69.0 \pm 8.9			70.8 \pm 9.3	
Dysplasia					
4	71.6 \pm 23.8	71.5 \pm 23.8	33.3%	72.4 \pm 26.1	71.8 \pm 25.4
5	70.2 \pm 25.4			71.1 \pm 26.4	
6	72.6 \pm 22.2			71.9 \pm 23.7	
Carcinoma					
7	97.8 \pm 41.8	117.5 \pm 57.5	48.9%	99.2 \pm 44.1	129.1 \pm 61.4
8	138.1 \pm 74.1			159.4 \pm 78.9	
9	116.6 \pm 44.4			128.7 \pm 61.0	
Area (μm^2)					
Normal					
1	55.2 \pm 11.8	47.7 \pm 11.3	23.7%	53.8 \pm 10.7	48.0 \pm 10.5
2	44.4 \pm 9.6			45.0 \pm 9.9	
3	43.5 \pm 8.4			45.3 \pm 8.9	
Dysplasia					
4	46.9 \pm 16.1	41.4 \pm 15.4	37.2%	45.7 \pm 17.2	41.6 \pm 16.0
5	40.3 \pm 14.9			41.2 \pm 14.0	
6	37.0 \pm 13.7			38.0 \pm 14.2	
Carcinoma					
7	70.6 \pm 22.9	73.3 \pm 34.8	47.5%	76.4 \pm 24.0	87.6 \pm 35.1
8	82.6 \pm 43.6			92.7 \pm 42.1	
9	66.7 \pm 28.3			78.6 \pm 30.3	

ticles depends to some extent on the type of fixative used. Some produce coarser clumping than others (Greisen, 1971). In this laboratory 10% neutral buffered formalin has been found to be the best fixative (Hellquist et al. 1975).

7 The processing method must be stable. By measuring lymphocytes from blocks embedded and stained at different times the reproducibility of the staining reaction could be controlled.

8 A consistent thickness of the sections is another important requirement. If the number of measurements is large enough the thickness of the sections can be regarded as a statistical variable and by using the mean any fluctuations in thickness will then be eliminated (Greisen, 1971).

RESULTS

1 Sections thickness 8 μm

Measurements on 75 interphase nuclei per sample (Table I).

Normal laryngeal squamous epithelium (Cases 1-3). A histogram showing the DNA content of normal epithelial nuclei and lymphocytes is presented in Fig. 1A. The mean DNA content of normal epithelial nuclei was 69.2 arbitrary units (A U). The range was fairly small, the coefficient of variation being 12.0%. The mean area of the nuclei was 47.7 μm^2 , with a coefficient of variation of 23.7%. Comparison of the DNA content and the area of the nuclei gave a coefficient of correlation of 0.56 (Table II).

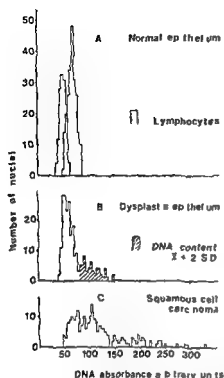


Fig 1 Frequency histograms of nuclear DNA absorbance (content) A normal epithelium B dysplastic epithelium C squamous cell carcinoma Section thickness $8\ \mu\text{m}$ = mean absorbance value for normal epithelium (69.2 U)

Dysplastic epithelium (Cases 4–6) The DNA distribution for nuclei of dysplastic epithelium differed from that for the normal nuclei (Fig 1B). The mean DNA content 71.5 AU was not increased but there was a large variability about the mean. The standard deviation was 23.8 AU, giving a coefficient of variation of 33.3%.

The mean area of the nuclei was $41.4\ \mu\text{m}^2$, and the coefficient of variation 37.2%.

The coefficient of correlation between the DNA content and the area of the nuclei was 0.79 (Table II).

Squamous cell carcinoma (Cases 7–9) The histogram for the distribution of the tumour nuclei by DNA content shows a wide range of ploidy class (Fig 1C). Although there is a tendency towards aneuploidy, there is a large class around the tetraploid value. Both the mean and the variation were increased with values of 117.5 AU and 48.9% respectively.

The mean nuclear area of $73.3\ \mu\text{m}^2$ implied a greater size than for either normal or dysplastic epithelium. The coefficient of variation was 47.5%. The coefficient of correlation for DNA content versus area was as high as 0.86 (Table II); this points to a close relationship between nuclear size and DNA content.

II Sections thickness $14\ \mu\text{m}$

Measurements on 15 interphase nuclei per sample (Table I).

Normal, dysplastic and carcinomatous epithelium (Cases 1–9) The area and DNA content of nuclei of the normal and dysplastic epithelium were about the same in the $14\ \mu\text{m}$ and the $8\ \mu\text{m}$ sections. On the other hand, the nuclear area in squamous cell carcinoma was increased.

III Lymphocytes (Fig 1A and Table III)

- Sections of thickness 8 and $14\ \mu\text{m}$ (Cases 1–9). Measurements on 15 lymphocytes per sample.
- Smears: (i) Three imprints. Measurements on 15 lymphocytes per sample. (ii) Blot. Measurements on 25 lymphocytes.

There was no difference in the mean DNA content for the 8 and $14\ \mu\text{m}$ sections. There was a significant difference between sections and smears.

Table II Coefficient of correlation in linear regression of nuclear DNA absorbance (content) and area (μm^2)

Case	Coefficient of correlation	
Normal		
1	0.57	0.56
2	0.55	
3	0.71	
Dysplasia		
4	0.86	0.79
5	0.85	
6	0.71	
Carcinoma		
7	0.85	0.86
8	0.89	
9	0.84	

Table III Mean values of the DNA absorbance (content) of lymphocytes

15 $\nabla n=25$

Histological sections		Smears	
8 $\mu m \Delta$	14 $\mu m \Delta$	Imprints Δ	Blood ∇
52.2	53.0	52.1	51.9
51.4	51.1	51.8	
51.5	53.0	51.0	
50.3	51.2		
50.6	52.2		
51.8	51.4		
52.0	51.2		
53.2	52.5		
51.5	53.2		
51.7	52.1	51.6	51.9

The mean DNA content for 8 μm sections, namely 51.7 A U, was taken as the reference the diploid value (2N)

DISCUSSION AND CONCLUSIONS

In histological grading of atypia is based on comparison with normal epithelium, and the evaluation contains a large subjective element. In order to achieve a higher level of objectivity in the classification a photometric evaluation of the DNA content and the variation in nuclear size was performed using a Leitz scanning photometer.

In photometric examination of histological sections it is necessary to identify undamaged nuclei that represent the relevant histopathological changes. Since it is very difficult to completely exclude nuclei that have been cut by the microtome photometry of histological sections is not a strictly quantitative method. It does however have the advantage that the examination can be performed on representative portions of the specimens.

Lymphocytes are considered to have a normal DNA content. In this study the value is 75% of the mean for normal epithelial nuclei. While this difference cannot at present be adequately accounted for, it is consistent

with the results reported by other investigators (Atkin & Richards, 1956, Atkin, 1964, Bader et al., 1960, Wagner et al., 1967). The photometric parameters of atypias were accordingly compared with those for normal mucosa. The mean DNA content of normal laryngeal epithelium, namely 69.2 A U (Cases 1-3) is referred to not as 2N but as \bar{x} .

The fact that the size of the nuclei in normal and dysplastic epithelium was not greater in the 14 μm than in the 8 μm sections suggests that the latter displayed no effect of cutting. Furthermore, the nuclei in dysplastic epithelium were essentially the same size as those in normal epithelium, a comparison can therefore be made. The carcinoma cell nuclei (Cases 7-9), on the other hand, were larger in 14 μm sections, and some of them may therefore have been cut in 8 μm sections, this may have affected the absorbance values (Table I, Fig. 1C).

When the DNA content exceeds the mean by more than twice the standard deviation—corresponding to 3.3 N—the nuclei were considered to be hyperchromatic. The absence of hyperchromatic nuclei in normal laryngeal epithelium (Fig. 1A) is consistent with the absence of DNA synthesis above the parabasal layer (Fabrikant & Cherry, 1969). In dysplastic and carcinomatous laryngeal epithelia on the other hand hyperchromatic nuclei are numerous (Fig. 1B, C) which is also found by Greisen (1975) and Giarelli et al. (1977).

So long as the mean DNA content does not fall significantly below its normal value an increase in the coefficient of variation implies the presence of nuclei with an elevated DNA content. Thus, another criteria of the hyperchromasia in a cell population may be the coefficient of variation of the DNA content. The coefficient of variation for the nuclear area was markedly higher for nuclei of dysplastic epithelium than for nuclei of normal epithelium. It would thus seem that the variability of both nuclear size and DNA content increases with the degree of atypia.

Comparison of each cell nucleus

AUTHOR INDEX

- Afzelius L E Hennksson N G and Wahlgren L Vertigo as Reflected by the Nystagmogram 123
- Allum J H I See Brandt Th Allum J H I and Dichgans J
- Alumets J See Uddman R Alumets J Densert O Håkansson R and Sundler F
- Andersen I See Torgussen W Haug F M S and Andersen I
- Andreasson L Harris S and Ivarsson A Pulse Volume Recordings in outer Ear Canal in Pulse Synchronous Tinnitus 741
- Anniko M and Möller A R A Physiological and Morphological Study of the Cochlea of the Rat Following Treatment with Atoxyl and Neomycin 201
- Anniko M Van der Water T R and Nordemar H Organ Culture of the 16th Gestation Day Mouse Labyrinth 52
- Axelsson H Carlsoo B Weibring J and Winblad H Aspergillosis of the Maxillary Sinus 303
- Bagger Sjöback D and Wersäll J Gentamicin induced Mitochondrial Damage in Inner Ear Sensory Cells of the Lizard *Calotes Versicolor* 35
- Balle V See Illum P and Balle V
- Bannister L H See Dodson H C Bannister L H and Douek E E
- Beck P See Thomsen J Beck P and Saxtrup Nielsen O
- Benedek Spat Enka Sialochemical Examinations in Non Tumorous Parotid Enlargements 276
- Bess F H See Humes L E and Bess F H
- Borg E Peripheral Vasoconstriction in the Rat in Response to Sound 155
- Brandt Th Allum J H J and Dichgans J Computer Analysis of Optokinetic Nystagmus of Peripheral Vestibular Origin 115
- Brown J J Brummett R E Meikle M B and Vernon J Combined Effects of Noise and Neomycin Cochlear Changes in the Guinea Pig 394
- Brummett R E See Brown J J Brummett R E Meikle M B and Vernon J
- Burton McKay C See Dennis K C Guth P S Givens G D and Burton McKay C
- Cabezudo Lucia M The Ultrastructure of the Basilar Membrane in the Cat 160
- Carenfelt C Lundberg C Nord C E and Wretling H Bacteriology of Maxillary Sinusitis in Relation to Quality of the Retained Secretion 798
- Carlsoo B See Axelsson H Carlsoo H Weibring J and Winblad H
- Cohen G M and Ferman C D The Development of Hair Cells in the Embryonic Chick's Basilar Papilla 342
- Collan Y See Palva T Horthling L Ylikoski and Collan Y
- Dennis K C Guth P S Givens G D and Burton McKay C Fluorescent Staining of Hair Cells with Ethidium Bromide 217
- Densert O See Uddman R Alumets J Densert O Håkansson R and Sundler F
- Dichgans J See Brandt Th Allum J H J and Dichgans J
- Dodson H C Bannister L H and Douek E E Further Studies of the Effects of Continuous White Noise of Moderate Intensity (70-80 dB SPL) on the Cochlea in Young Guinea Pigs 195
- Douek E E See Dodson H C Bannister L H and Douek E E
- Dubno J R See Popelka G R and Dubno J R
- Eccles R The Central Rhythm of the Nasal Cycle 464

- Elbrønd, O See Vinther, B and Elbrønd, O
- Englund, A See Engzell, U, Englund, A and Westerholm, P
- Engzell, U, Englund, A and Westerholm, P Nasal Cancer Associated with Occupational Exposure to Organic Dust 437
- Fermin, C D See Cohen, G M and Fermin C D
- Firbas, W The Efferent Innervation in the Region of Inner Hair Cells in the Organ of Corti 109
- Fredrickson, J M See Rubin, A M, Liedgren S R C, Ödkvist L M Milne A C and Fredrickson, J M
- Fritze, W A Computer controlled Binaural Balance Test 89
- Givens, G D See Dennis, K C, Guth, P S, Givens, G D and Burton McKay C
- Grontoft, O, Hellquist H, Olofsson, J and Nordstrom, G The DNA Content and Nuclear Size in Normal Dysplastic and Carcinomatous Laryngeal Epithelium 473
- Gundersen, T, Skarstein, Ø and Sikkeland, T A Study of the Vibration of the Basilar Membrane in Human Temporal Bone Preparations by the Use of the Mossbauer Effect 225
- Givens, G D See Dennis, K C, Guth, P S, Givens, G D and Burton McKay C
- Hamrick, P E See Konishi, T and Hamrick, P E
- Hamrick, P R See Konishi, T, Hamrick, P R and Walsh P J
- Harris, S See Andreasson, L, Harris, S and Ivarsson A
- Haug, F M S See Torjussen W, Haug, F-M S and Andersen I
- Hellquist, H See Grontoft, O, Hellquist, H, Olofsson, J and Nordstrom, G
- Henniksson, N G See Afzelius, L-E, Henniksson N G and Wahlren L
- Henry, K R and Lepkowski, C M Evoked Potential Correlates of Genetic Progressive Hearing Loss 466
- Hortling L See Palva, T, Hortling, L, Ylikoski, J and Collan, Y
- Hoshino, T, Kato I, Kodama, A and Suzuki, H Sudden Deafness in Relapsing Polychondritis, A Scanning Electron Microscopy Study 418
- Hubbard, D G See Wright, C G and Hubbard D G
- Humes, I E and Bess F H A Test Battery Approach to the Investigation of Susceptibility to Temporary Threshold Shift 385
- Håkanson R See Uddman, R, Alumets, J, Denbert O, Håkanson R and Sundler, F
- Ilum P and Balle V Immunoglobulins in Nasal Secretions and Nasal Mucosa in Perennial Rhinitis 135
- Ise, I See Kaneko Y, Terayama Y, Kawamoto, K, Kasajima, K, and Ise, I
- Ise, I See Kusakan, J, Kambayashi, J, Ise, I and Kawamoto, K
- Ivarsson A, See Andreasson L, Harris, S and Ivarsson, A
- Jackson D G and Lim D L Fine Morphology of the Advancing Front of Cholesteatoma Experimental and Human 71
- Jauhainen T See Palva T, Jauhainen, T, Sjöblom, C-J and Ylikoski J
- Jokipii, A M M See Karma P, Jokipii, L, Ojala K and Jokipii A M M
- Jokipii, L See Karma P, Jokipii L, Ojala, K and Jokipii, A M A
- Jungert, S See Renvall U, Liden G, Jungert, S and Nilsson, F
- Kaaber, K See Winkel OJ, Mørk Hansen M, Kaaber K and Rozarth, K
- Kambayashi J See Kusakan J, Kambayashi, J, Ise, I and Kawamoto K
- Kaneko Y, Terayama Y, Kawamoto K, Kasajima, K, and Ise, I Single Cell Layer Membrane Covering the Degenerated Cochlear Duct after Perilymphatic Perfusion of Streptomycin 375
- Karma, P, Jokipii L, Ojala K and Jokipii, A M M Bacteriology of the Chronically Discharging of Middle Ear 110

- Kasajima K See Kaneko Y Terayama Y Kawamoto K Kasajima K and Ise I
- Kato I See Hoshino T Kato I Kodama A and Suzuki H
- Kawamoto K See Kaneko Y Terayama Y Kawamoto K Kasajima K and Ise I
- Kawamoto K See Kusakari J Kambayashi J Ise I and Kawamoto K
- Kodamar A See Hoshino T Kato I Kodama A and Suzuki H
- Konishi T and Hamrick P E Ion Transport in the Cochlea of Guinea Pig II
Chlonde Transport 176
- Konishi T Hamrick P R and Walsh P J Ion Transport in Guinea Pig
Cochlea 22
- Kusakari J Kambayashi J Ise I and Kawamoto K Reduction of the Endo-
cochlear Potential by the new Loop Diuretic Bumetanide 336
- Lepkowski C M See Henry K R and Lepkowski C M
- Liden G See Renvall U Liden G Jungert S and Nilsson E
- Liedgren S R C See Rubin A M Liedgren S R C Odkvist L M Milne
A C and Fredrickson J M
- Lim D L See Jackson D G and Lim D L
- Lundberg C See Carenfelt C Lundberg C Nord C E and Wretling B
- Magnusson B Tubal Closing Failure in Retraction Type Cholesteatoma and Ad-
hesive Middle Ear Lesions 408
- Margolis R H Osguthorpe J D and Popelka G R The Effects of Experi-
mentally Produced Middle Ear Lesions on Tympanometry in Cato 428
- Meikle M R See Brown J J Brummett H E Meikle M D and Vernon J
- Meistrup-Larsen K I Mygind N Thomsen J Sorensen H and Vesterhauge
S Oral Norephedrine in the Treatment of Acute Otitis Media 248
- Milne A C See Rubin A M Liedgren S R C Odkvist L M Milne A C
and Fredrickson J M
- Milne A C See Schwarz D W F Tomlinson R E and Milne A C
- Mogensen Chr and Tos M Experimental Surgery on the Nose 289
- Monoka H See Tachibana M Saito H Yananichi I and Monoka H
- Møller A Responses of Auditory Nerve Fibres to Noise Stimuli Show Cochlear
Nonlinearities 1
- Møller A R See Anniko M and Møller A R
- Mørk Hansen M See Winkel O Mørk Hansen M Kaaber K and Rozarth K
- Mygind N See Meistrup Larsen K I Mygind N Thomsen J Sorensen H
and Vesterhauge E
- Nilsson E See Renvall U Liden G Jungert S and Nilsson E
- Nord C E See Carenfelt C Lundberg C Nord C E and Wretling B
- Nordemar H See Anniko M Van der Water T R and Nordemar H
- N
(
(
(
- Osguthorpe J D See Margolis R H Osguthorpe J D and Popelka G R
- Palva T Jauhainen T Sjoblom C J and Ylikoski J Diagnosis and Surgery of
Acoustic Tumours 233
- Palva T Hortling L Ylikoski J and Collan Y Viral Culture and Electron
Microscopy of Ganglion cells in Meniere's Disease and Bell's Palsy 269
- Popelka G R and Dubno J R Comments on the Acoustic reflex Response for
Bone-conducted Signals
- Popelka G R See Margolis R H Osguthorpe J D and Popel

- Elbrond, O See Vinther, B and Elbrond, O
- Englund, A See Engzell, U, Englund, A and Westerholm, P
- Engzell, U, Englund, A and Westerholm, P¹ Nasal Cancer Associated with Occupational Exposure to Organic Dust 437
- Fermin, C D See Cohen, G M and Fermin, C D
- Firbas, W The Efferent Innervation in the Region of Inner Hair Cells in the Organ of Corti 309
- Fredrickson, J M See Rubin, A M, Liedgren, S R C, Ödkvist, L M, Milne, A C and Fredrickson, J M
- Fritze, W A Computer controlled Binaural Balance Test 89
- Givens, G D See Dennis, K C, Guth, P S, Givens, G D and Burton, McKay C
- Grontoft, O, Hellquist, H, Olofsson, J and Nordstrom, G The DNA Content and Nuclear Size in Normal Dysplastic and Carcinomatous Laryngeal Epithelium 473
- Gundersen, T, Skarstein, Ø and Sikkeland, T A Study of the Vibration of the Basilar Membrane in Human Temporal Bone Preparations by the Use of the Mossbauer Effect 223
- Givens, G D See Dennis, K C, Guth, P S, Givens, G D and Burton, McKay C
- Hamrick, P E See Konishi, T and Hamrick, P E
- Hamrick, P R See Konishi, T, Hamrick, P R and Walsh, P J
- Harnis, S See Andréasson, L, Harnis, S and Ivarsson, A
- Haug, F M S See Torjussen, W, Haug, F-M S and Andersen, I
- Hellquist, H See Grontoft, O, Hellquist, H, Olofsson, J and Nordstrom, G
- Henriksson, N G See Afzelius, L E, Henriksson, N G and Wahlren, L
- Henry, K R and Lepkowski, C M Evoked Potential Correlates of Genetic Progressive Hearing Loss 366
- Hortling, L See Palva, T, Hortling, L, Ylikoski, J and Collan, Y
- Hoshino, T, Kato, I, Kodama, A and Suzuki, H Sudden Deafness in Relapsing Polychondritis A Scanning Electron Microscopy Study 418
- Hubbard, D G See Wright, C G and Hubbard, D G
- Humes, L E and Bess, F H A Test Battery Approach to the Investigation of Susceptibility to Temporary Threshold Shift 385
- Håkanson, R See Uddman, R, Alumets, J, Densert, O, Håkanson, R and Sundler, F
- Illum, P and Balle, V Immunoglobulins in Nasal Secretions and Nasal Mucosa in Perennial Rhinitis 135
- Ise, I See Kaneko, Y, Terayama, Y, Kawamoto, K, Kasajima, K and Ise, I
- Ise, I See Kusakari, J, Kambayashi, J, Ise, I and Kawamoto, K
- Ivarsson, A See Andréasson, L, Harnis, S and Ivarsson, A
- Jackson, D G and Lim, D L Fine Morphology of the Advancing Front of Cholesteatoma Experimental and Human 71
- Jauhiainen, T See Palva, T, Jauhiainen, T, Sjöblom, C-J and Ylikoski, J
- Jokipii, A M M See Karma, P, Jokipii, L, Ojala, K and Jokipii, A M M
- Jokipii, L See Karma, P, Jokipii, L, Ojala, K and Jokipii, A M M
- Jungert, S See Renvall, U, Liden, G, Jungert, S and Nilsson, E
- Kaaber, K See Winkel, O, Mørk Hansen, M, Kaaber, K and Rozarth, K
- Kambayashi, J See Kusakari, J, Kambayashi, J, Ise, I and Kawamoto, K
- Kaneko, Y, Terayama, Y, Kawamoto, K, Kasajima, K, and Ise, I Single-Cell Layer Membrane Covering the Degenerated Cochlear Duct after Perilymphatic Perfusion of Streptomycin 375
- Karma, P, Jokipii, L, Ojala, K and Jokipii, A M M Bacteriology of the Chronically Discharging of Middle Ear 110

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Fluorescent Staining of Hair Cells with Ethidium Bromide	217
Ganglion Cells in Meniere's Disease and Bell's Palsy, Viral Culture and Electron Microscope of	269
Genetic Progressive Hearing Loss Evoked Potential Correlates	366
Gentamicin Ototoxicity, a Prospective Study	212
Head Rotation in the Pigeons Posterior Cerebellum Directional Specificity for	260
Heavy Metals in Nasal Mucosa of Nickel Exposed Workers and of Controls Concentration and Distribution, Studied with Atomic Absorption Spectrophotometric Analysis and with Timm's Sulphide Silver Method	449
Immunoglobulins in Nasal Secretions and Nasal Mucosa in Perennial Rhinitis	135
Immunopathological Description of Tonsils after a Peritonsillar Abscess	
Ion Transport in Guinea Pig Cochlea	22
Membranous Systems of the Strial and External Sulcus Cells Differential Effects of Primary Fixation with Glutaldehyde and Osmium	56
Middle Ear Lesions on Tympanometry in Cats, the Effects of Experimentally Produced	428
Middle Ear Lesions Tubal Closing Failure in Retraction Type Cholesteatoma and Adhesive	408
Nasal Cancer Associated with Occupational Exposure to Organic Dust	437
Nasal Cycle, the Central Rhythm	464
Nasal Mucociliary Function during Penicillin Treatment	132
Nasal Mucosa and Tracheobronchial Wall, Occurrence and Distribution of Vpn Nerves	443
Noise Stimuli Show Cochlear Nonlinearities Responses of Auditory Nerve Fibres	1
Non Tumorous Parotid Enlargements, Sialochemical Examinations	276
Nystagmogram Vertigo as Reflected by	123
Optokinetic Nystagmus of Peripheral Vestibular Origin Computer Analysis	115
Organ Culture of the 16th Gestation Day Mouse Labyrinth	52
Oto- and Nephrotoxicity, Tissue Levels of Kanamycin in Correlation with	9
Otoconial Membranes Observations from Human Infants	185
Ototoxicity a Possible Involvement of Acidic Glycoaminoglycans in Kanamycin	15
Patulous Eustachian Tube	401
Pulse Synchronous Tinnitus Pulse Volume Recordings in outer Ear Canal	241
Reduced Middle Ear Pressure Longterm Observation of Ears	104
Reissner's Membrane Following Chemical Sympathectomy with 6-Hydroxydopa mine Glycogen Accumulation	314
Response to Sound Peripheral Vasoconstriction in the Rat	155
Sonotubometry	93
Sudden Deafness in Relapsing Polychondritis a Scanning Electron Microscopy Study	418
Surgery of Acoustic Tumours and Diagnosis of	233
Temporary Threshold Shift a Test Battery Approach to the Investigation of Susceptibility	385
The Mucociliary Activity of the Upper Respiratory Tract	283
Vestibulospinal Neurons Labyrinthine and Somatosensory Convergence	251
Vibration of the Basilar Membrane in Human Temporal Bone Preparations by the Use of the Mossbauer Effect	225
Vocal Function the Neuromuscular Phonatory Control System	142
White Noise of Moderate Intensity on the Cochlea in Young Guinea Pigs	195

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Conferences and Meetings

- 1978, Nov 23-26 Course on Septoplasty microsurgical aspects and endonasal approach of the pterigomaxillary fossa and annexed cavities" will be held in Barcelona
 Addr Secretary of the Cours, Muntaner, 366-3°, Barcelona 6 Spain
- 1979 Jan 15-19 The Fourteenth Annual Otolologic Surgery Course will be held at The Ear Research Institut in Los Angeles Addr F H Linthicum, Jr M D, Ear Research Institute, 256 South Lake Street, Los Angeles California 90057, USA
- 1979, March 4-9 Department of Otolaryngology, University of Toronto announces Winter Meeting 1979—Mont Trembland Lodge Addr Dr William Crysdale Suite 6126, 555 University Avenue, Toronto Canada M5G 1X8
- 1979 April 29-May 4 Third International Symposium on Plastic and Reconstructive Surgery of the Head and Neck to be held in New Orleans Addr Jack R Anderson, M D, 1111 Tulane Avenue, (Ste 322) New Orleans Louisiana 70112, USA
- 1979 April 1-6 The Asia Oceania Association of Oto-Rhino-Laryngological Societies Fourth Asia Oceania Congress to be held in Sydney, Australia Addr The Congress Secretariat GPO Box 2609, Sydney, NSW, Australia 2001
- 1979 April 22-28 The Department of Otolaryngology, University of Nijmegen the Netherlands, announces the 14th Post Graduate Course in Ear Surgery Addr Dr W F B Brinkman Department of Otolaryngology Philips van Leydenlaan 15, Nijmegen the Netherlands
- 1979, May 21-23 The Second International Symposium on Otitis Media with Effusion will be held at Ohio State University, Columbus, Ohio Addr David J Lim, M D, Department of Otolaryngology Ohio State University Hospitals, 456 Clinic Drive, Columbus, Ohio 43210 USA

